CANADIAN JOURNAL OF RESEARCH

VOLUME 21 APRIL, 1943 NUMBER 4

CONTENTS

SECTION C.—BOTANICAL SCIENCES

SECTION D.—ZOOLOGICAL SCIENCES

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

Publications and Subscriptions

The Canadian Journal of Research is issued monthly in four sections, as follows:

A. Physical Sciences
B. Chemical Sciences
C. Botanical Sciences
D. Zoological Sciences

For the present, Sections A and B are issued under a single cover, as also are Sections C and D, with separate pagination of the four sections, to permit separate binding, if desired.

Subscription rates, postage paid to any part of the world are as follows:

	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
C and D	2.50	0.50
Four sections, complete	4.00	—

The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

National Research Council, Ottawa, Canada.

Notice to Contributors

Fifty reprints of each paper are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 21, SEC. C. APRIL, 1943 NUMBER 4

VARIATION IN NUMBERS OF BACTERIA IN TWO PLOTS[1]

BY NORMAN JAMES[2] AND MARJORIE L. SUTHERLAND[3]

Abstract

An experiment was designed to study the combined effect of changes in moisture, dates of sampling, and numbers of colonies per plate on the estimated number of bacteria in the soil of two plots. The data represent 30 replicate estimates on a wheat plot and 30 on a fallow plot, each made from a different dilution on each of 19 dates of sampling from May 21 to September 27, together with moisture determinations for each plot on each date. The statistical treatment consists of a combination of correlation and analysis of variance studies. The results are presented graphically, and in the usual form of analysis of variance. They demonstrate the presence of a basic population in each plot which responds differently to moisture and time in different dilutions.

Introduction

Recent reports from this laboratory (1 — 4) on plate counts of bacteria in soil show clearly that a given estimate must be considered in relation to several factors before it can be interpreted in terms of agricultural practice.

1. The bacterial population in soil responds strongly to changes in moisture. A difference of 1% moisture in a given plot is associated with a change of about 1,000,000 bacteria per gram of soil.

2. Numbers of bacteria vary in a regular manner during the crop season. This variation is independent of changes in moisture.

3. The kind of crop on the plot sampled influences the rate of change in the population in response to differences in both moisture and date of sampling.

4. The number of colonies developing on the plates in the laboratory influences the estimate of the population. An estimate based on a dilution yielding 120 colonies per plate is significantly lower than one from the same sample based on another dilution yielding 20 colonies per plate.

The first three factors—moisture, time, and crop—influence the number of bacteria in the soil of a given plot and cause variation in the fourth factor—numbers of colonies per plate—in a series of determinations from the plot. Obviously, the variation in estimates resulting from the four factors and their

[1] *Manuscript received in original form November 16, 1942, and as revised, December 23, 1942.*

Contribution from the Department of Bacteriology and Animal Pathology, The University of Manitoba, Winnipeg, Man., with financial assistance from the National Research Council of Canada. Adapted from a thesis submitted by Miss Sutherland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] *Associate Professor of Bacteriology.*

[3] *Research Assistant in Bacteriology.*

interactions are so great that comparisons made on the basis of raw data are misleading.

It should be noted, of course, that while a consideration of these factors indicates the inadequacy of estimates based on raw plate count data, information regarding them may be of direct value in relation to a specific factor studied and/or in error control. Changes in estimates associated with differences in dates of sampling from a cropped plot may be characteristic for that crop and, as such, should be recorded. The same holds true for differences in moisture or any other environmental factor. Hence, the population response to these factors is of practical value in the interpretation of data. Likewise, changes in estimates related to differences in numbers of colonies on plates may represent definite differences in the populations on the plates. Or, they may indicate conditions associated wholly with differences in the environment in the plates. In the latter case they would merely add to the error of the experiment.

In order to obtain additional detailed information on these points an experiment was conducted with a fallow and a wheat plot in the summer of 1940. The design of the experiment was based on the hypothesis that a given plot has a basic population, whose equilibrium is constantly shifting with changes in environmental factors. The basic population and the responses to these factors can be estimated by the plate count method. The estimate, however, is influenced by the number of colonies on the plates counted.

The evidence supporting this hypothesis involves studies on individual environmental factors and on numbers of colonies per plate. This experiment was an attempt to study the combined effect of the various factors previously considered in relation to the basic population and its responses to changes in environment as reflected by different dilutions.

Procedure

Two one-hundredth acre plots, separated by a three foot space, were used in this study. One was fallow, and the other was seeded to wheat. Both plots were sampled at approximately weekly intervals from May 21 to September 27. On each date of sampling duplicate samples were taken from each plot. Each sample was a composite of six cores of surface soil 6 in. $\times$ $1\frac{1}{4}$ in., each core being taken at a point in the plot predetermined by randomization. A different randomization was used for each sample.

In the laboratory each sample was passed through a $\frac{1}{4}$ in. mesh screen by means of slight pressure and mixed for 10 min. in a mechanical mixer. A 50 gm. portion was oven-dried overnight at 104° C. for a moisture determination. A second 50 gm. portion was suspended in 2500 ml. of water and agitated on a to-and-fro mechanical shaker for 10 min. This original 1/50 dilution was agitated as portions were withdrawn to prepare 30 higher dilutions. The final dilutions were chosen in order that, theoretically, a population of 30,000,000 per gram of oven-dried soil would give counts starting at 10 colonies per plate from Dilution 1 and increasing by 10 per dilution

Dilution number	Parts water to 1 part of soil	Theoretical number of colonies per plate	
		Population of 15,000,000	Population of 30,000,000
1	3,000,000	5	10
2	1,500,000	10	20
3	1,000,000	15	30
4	750,000	20	40
5	600,000	25	50
6	500,000	30	60
7	428,571	35	70
8	375,000	40	80
9	333,333	45	90
10	300,000	50	100
11	272,727	55	110
12	250,000	60	120
13	230,769	65	130
14	214,286	70	140
15	200,000	75	150
16	187,500	80	160
17	176,470	85	170
18	166,667	90	180
19	157,895	95	190
20	150,000	100	200
21	142,857	105	210
22	136,364	110	220
23	130,435	115	230
24	125,000	120	240
25	120,000	125	250
26	115,384	130	260
27	111,111	135	270
28	107,142	140	280
29	103,448	145	290
30	100,000	150	300

up to 300 colonies per plate from Dilution 30. The actual number of colonies per plate from any sample, of course, would depend on the population sampled. The 30 dilutions were prepared according to the following plan.

Duplicate plates were prepared from each dilution. Thus, on one date of sampling two plates from each of two samples were prepared from each of 30 different dilutions from the two plots. Waksman's sodium albuminate agar was used and incubation was at 26° C. for six days. Estimates from the four plates were averaged to give the bacterial population for each dilution. This procedure was repeated 19 times between May 21 and September 27. Dates of sampling were the same for the two plots, whereas the moisture content of the soil in the two plots differed and changed with dates of sampling. The dates and moisture data follow.

Dilution No.	Date	Moisture, %	
		Fallow	Wheat
1	May 21	33.58	32.49
2	May 25	30.11	30.65
3	May 31	28.27	22.55
4	June 7	29.94	29.03
5	June 15	34.59	31.82
6	July 2	28.50	25.09

Dilution No.	Date	Moisture, %	
		Fallow	Wheat
7	July 5	27.71	21.39
8	July 12	24.88	21.07
9	July 20	23.18	20.05
10	July 26	21.54	20.19
11	Aug. 1	22.97	22.10
12	Aug. 9	24.72	27.00
13	Aug. 15	23.95	29.10
14	Aug. 23	21.89	23.06
15	Aug. 29	21.62	22.61
16	Sept. 6	28.60	30.65
17	Sept. 13	24.22	25.22
18	Sept. 21	24.97	27.75
19	Sept. 27	23.33	24.38

Treatment of Data

The data are reduced to a series of charts, each one a graphic presentation of definite points derived from the data. This is followed by a brief consideration of the analysis of variance.

Original Data

The data for each plot were graphed in three dimensions, the X axis representing days from the beginning of the experiment, the Y axis, Dilutions 1 to 30 and the Z axis, estimates of bacteria obtained by the procedure outlined. This was done to show general variation in the original data. The graphs were photographed at an angle that gives a better view of the variation between dates of sampling, or what has been referred to as seasonal fluctuation, than that between dilutions. The estimates from different dilutions on any date differ only because of differences in the number of colonies on plates, and for this illustration may be considered as replicates. They are shown in Fig. 1.

The data are highly variable and apparently quite different in the two plots. On the basis of previous studies (2, 3) a portion of this variability should be explainable by response to changes in moisture and time. The amount of moisture differs in the two plots and its effect on the population is likely to be different because of the crop in one plot. However, the data as presented give no indication of what the responses to moisture and time are, and obviously are so erratic that a simple comparison of the populations in the two plots would be meaningless and misleading.

Distribution of Deviations from the Means for Each Dilution

Again since estimates from the 30 dilutions on one date may be considered replicates, and further since the 19 estimates from any one dilution over the season are much more variable than estimates from the 30 different dilutions on one date, graphs were prepared to determine whether the distribution of the variation within dilutions is the same for all dilutions from each plot and to detect differences between plots. Deviations of the 19 individual

estimates from the mean for each dilution were recorded and plotted as shown in Fig. 2, the negative deviations being represented by shaded squares and the positive by blanks.

The distribution of deviations is distinctly different in the two sets of data. Most of the negative values are concentrated between sampling dates 1 and 10 in the fallow, and between 8 and 19 in the wheat plot data. In general, all of the dilutions in either chart show the same grouping of positive and negative deviations. This is accepted as evidence of a difference between the bacterial populations in the two plots. However, it does not justify an attempt to define this difference or interpret the data until they have been submitted to further analysis.

Correlations and Regressions

Multiple correlation of bacteria with moisture and time was carried out on the data for within each of the 30 dilutions from the wheat plot. However, since the time effect was not significant in the fallow plot data, the simple bacteria–moisture correlation was used. The data provide a regression equation for each dilution. From it, the populations at seven selected combinations of moisture and time were calculated—at 65 days with each of 20, 25, 30, and 35% moisture; and at 25% moisture with each of 1, 65, and 130 days. The four estimates at 65 days for each plot were plotted on the Y axis directly above Dilution 1 and joined by a light line on the moisture surfaces—65 days, shown in Fig. 3. This procedure was repeated for each of the other dilutions. Similarly, the three estimates at 25% moisture from each dilution in the wheat plot data and the one estimate at 25% moisture in the fallow plot data were plotted and appear in the crop surfaces—25% moisture. Thus, each vertical line is a regression line representing the change in numbers of bacteria estimated from one dilution at 65 days over a range of 20 to 35% moisture on the moisture surfaces, or at 25% moisture over a range of 1 to 130 days in the wheat crop surface.

Further, the 30 different dilution estimates for the populations at each of the seven selected combinations of moisture and time were correlated with dilution numbers 1 to 30; and a highly significant polynomial curve was found to express the relationship in each case. These curves, which appear in Fig. 3, show differences between dilutions—differences that are associated with differences in numbers of colonies on plates.

1. In either plot the moisture and crop surfaces are quite different, indicating that the bacterial response to changes in moisture differs from that to changes in time.

2. The moisture and crop surfaces for the wheat plot data differ from those for the fallow plot data, which may be associated with factors related to the developing crop.

3. The differences between the moisture surfaces for the two plots represent differences in size of response, as reflected by different dilutions, more so than differences between population levels in the two plots. The differences in

size of response from the different dilutions are not due wholly to random variation, as is made clear by the highly significant polynomial curve which expresses the relationship between estimates and dilution numbers at each of the four moisture levels. That is, the number of colonies on plates appears to have a strong influence on the estimate of the population—the larger the number of colonies, the smaller the estimate. The amount of reduction in estimates appears to vary with the moisture level, and is different in the wheat and fallow plots at each moisture level.

4. There is a strong population response to change in time in the wheat plot data, whereas in the fallow data there is no significant response to time, as is shown by the single line on the fallow plot crop surface. In each plot there is evidence that the population estimates decrease with increasing dilution numbers, and consequently with increasing numbers of colonies on plates. However, in contrast to that shown for the response to moisture in either plot, the size of the response to time in the wheat plot data does not appear to change appreciably with increasing numbers of colonies on plates.

A consideration of these points makes it obvious that a simple comparison based on original estimates of numbers of bacteria in the two plots is of little value. To be worth while a comparison should involve, at least, the response of the population to:

(*a*) Differences in moisture,

(*b*) Differences in time or dates of sampling,

(*c*) Differences in dilution and consequently in number of colonies per plate. It should be made at the same conditions of moisture, time, and dilution. It will differ with different combinations of these factors. On this basis, a comparison of the numbers of bacteria in the fallow and wheat plots gives the following.

Source of estimate, days at 25% moisture	No. bacteria, millions		Source of estimate, days at 25% moisture	No. bacteria, millions	
	Fallow	Wheat		Fallow	Wheat
Dilution 1			*Dilution 20*		
1	22.3	18.4	1	16.0	13.5
65	22.3	23.5	65	16.0	19.5
130	22.3	27.0	130	16.0	25.3
Dilution 10			*Dilution 30*		
1	16.0	15.0	1	15.5	14.8
65	16.0	21.0	65	15.5	19.2
130	16.0	27.5	130	15.5	22.3

Further, plate count estimates vary in a regular manner in response to changes in moisture and time. The size of this response differs in the different dilutions used in obtaining estimates, and apparently is determined by the number of colonies developing on the plates. Consequently, in each plot

there is a definite population level for a given combination of moisture, time, and dilution. A single estimate of this population differs from the actual population level because of random sampling from the field and at every step in the laboratory procedure. Since there is no way of determining the probable error of a single estimate, nor from that estimate a means of predicting the population under any other set of conditions, a single estimate of the population in a plot has little practical value. This is true particularly when it is known that samples obtained from different sources on one date contain different amounts of moisture, and this difference in moisture as well as in other environmental factors produces different numbers of colonies on plates, even if one system of diluting is used for all samples. Accordingly, a single estimate should not be used as a basis for comparing the population in one plot with that in another.

Distribution of Deviations from the Regressions Within Each Dilution

The unexplained variation within each dilution, or the deviations from the multiple regression of bacteria on moisture and days in the wheat plot data and from the simple regression of bacteria on moisture in the fallow plot data, were distributed in the manner described for the data for Fig. 2. These deviations appear in Fig. 4, the negative deviations again being represented by shaded squares and the positive by blanks.

In contrast to those shown in Fig. 2, there is no consistent grouping of positive or of negative deviations within the various dilutions. They appear to be distributed at random in each dilution. This apparently random distribution of deviations from the regressions for each dilution indicates that these regressions remove trends from the data; and that the residual variation within each dilution may be attributed largely to random sampling at the various steps in the procedure. When the chart for either plot is considered crosswise it is apparent that on certain dates most of the deviations are positive, while on other dates most are negative. This is to be expected since on any date estimates made on the basis of 30 different dilutions represent replicate estimates of the same population, and estimates from one plot on different dates would vary because of random sampling from the plot and at every step in the laboratory procedure.

Residual Variation

While Fig. 4 shows that there is variation within each dilution it does not indicate the extent of this variation from the population mean in any dilution. Nor does it show differences among population levels in different dilutions. In order to show the variation in the data not explained by the regressions of bacteria on moisture and days and the polynomial curves, expressing the relationship between estimates at constant conditions and dilution numbers, a new set of data for each plot was prepared. To obviate the necessity of working with both positive and negative values the population estimated from Dilution 15 at 25% moisture and 65 days was used as a base for each plot. This gave a base of 20.0787 millions bacteria per gram in the wheat

plot, and of 16.1518 in the fallow plot. Naturally these bases would have been different had another combination of moisture, time, and dilution been selected. The deviations from the polynomial curves at 25% moisture and 65 days were added to these bases to produce a series of 30 dilution levels for each plot, freed of the effect of differences in numbers of colonies on plates.

Then, in the wheat plot data each of the 19 deviations from the multiple regression of bacteria on moisture and days, representing the unexplained variation within Dilution 1, was added to the new dilution level for Dilution 1. That is, the random dilution level for Dilution 1 and the random variation within the dilution were combined to give a new set of 19 estimates for Dilution 1. This procedure was repeated for each of the other dilutions. The fallow plot data were treated in the same way except for the use of deviations from the simple regressions of bacteria on moisture. That is, the data represent (*a*) the variation within each dilution not explained by the perpendicular lines in Fig. 3, added to (*b*) the variation between dilutions at 25% moisture and 65 days which is not explained by the correlation of dilution estimates with dilution numbers, also shown in Fig. 3, and the sum attached to (*c*) an arbitrary base for each plot calculated from Dilution 15 for the population at 25% moisture and 65 days. The new estimates were plotted in three dimensions to show the unexplained variation in the data. They are presented in Fig. 5 for comparison with Fig. 1.

Fig. 5 is distinctly different from Fig. 1. The data for each plot are less variable, and do not differ widely in the two plots except for a somewhat higher population level in the wheat plot. They show no consistent trends. That is, the population as a whole does not appear to change from May to September, or from Dilution 1 to Dilution 30. This is accepted as strong evidence that once the responses to changes in moisture, time, and dilution have been removed from the data there is a basic population level in each plot. Estimates of that population would vary at random because of random sampling variation in the field and at every step in the laboratory procedure. It confirms the belief that what has been accepted as seasonal fluctuation represents failure to interpret the population response to changes in environment.

Analysis of Variance

The series of illustrations represents an attempt to present in simple form a large amount of data that might have been shown in terms of the analysis of variance procedure. The relationship is indicated in the following.

Total Sum of Squares—569 Degrees of Freedom

Fig. 1 shows the variation in the raw data for each plot. The total sum of squares for the experiment is calculated from these data. It consists of two portions:

(*a*) The sum of squares for differences between dilutions.

(*b*) The sum of squares for differences within dilutions.

Between Dilutions—29 Degrees of Freedom

The means for moisture differ in the two plots—fallow, 26.25% and wheat, 25.59%, whereas the means for days are the same—66.99 days in each plot. Further, the rate of increase in the population per unit change in moisture, and also the rate per unit change in time differs in the plots. Consequently, the populations should be compared at constant conditions of moisture and time. Since 25% moisture and 65 days are close to the actual means for moisture and days and also, since these figures fit well into the development

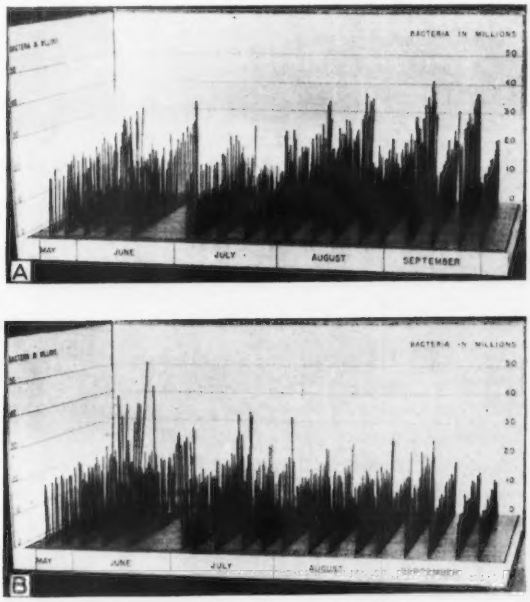


FIG. 1. *Variation in numbers of bacteria with differences in dates of sampling and in dilutions—original data. May 21 to Sept. 27—left to right; Dilutions 30 to 1—from front to back; bacteria in millions as vertical lines. A—wheat plot, B—fallow plot.*

of Fig. 3, the sum of squares for the variation between dilutions in each plot was calculated from dilution estimates at 25% moisture and 65 days. These estimates are represented in the illustrations by rows of 30 points on the perpendicular lines close to the curves calculated for 25% moisture and 65 days on the moisture and crop surfaces. This sum of squares, likewise, consists of two portions.

(*a*) *Regression of Bacteria on Dilution Numbers*

Fallow plot—4 degrees of freedom. Wheat plot—2 degrees of freedom.

A polynomial curve expresses this regression in each case. It is shown, in Fig. 3, as a heavy line labelled 25% on the moisture surfaces and 65 or 1 to 130 days on the crop surfaces.

(*b*) *Deviations from the Regression*

Fallow plot—25 degrees of freedom. Wheat plot—27 degrees of freedom.

The deviations are shown, in Fig. 3, as the distances of the points on the perpendicular lines from the curve. This sum of squares is the error against which the regression is tested for significance.

Within Dilutions—540 Degrees of Freedom

The distribution of the deviations responsible for the sum of squares for within dilutions in each plot is shown in Fig. 2. This sum of squares consists of three portions.

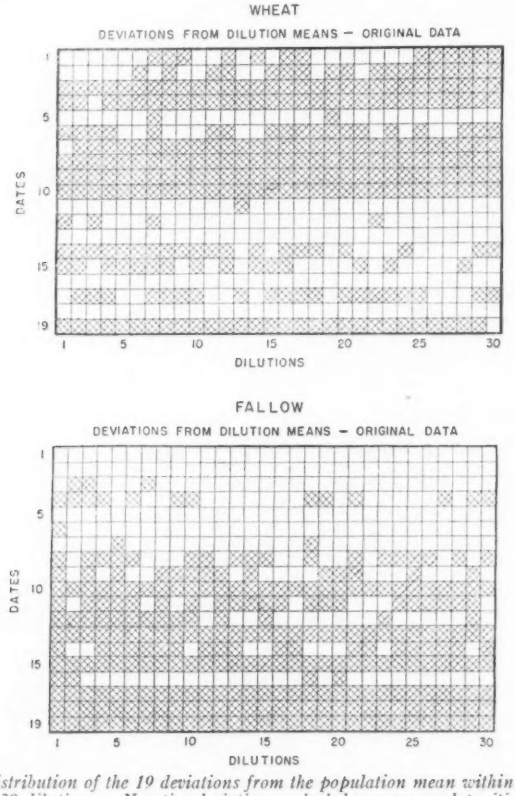


FIG. 2. *Distribution of the 19 deviations from the population mean within each dilution, shown for the 30 dilutions. Negative deviations—shaded squares, and positive deviations—blanks.*

(*a*) *Correlation of Bacteria and Moisture, with Days Constant*

Degrees of freedom, 30. These degrees of freedom are divided as follows:

(1) Average regression of bacteria on moisture, independent of days—1 degree of freedom.

(2) Differences between dilution regressions of bacteria on moisture, independent of changes in time—29 degrees of freedom. The 30 dilution regressions are represented by the perpendicular lines on the moisture surfaces in Fig. 3.

(*b*) *Correlation of Bacteria and Days, with Moisture Constant*

Wheat plot only—30 degrees of freedom. These degrees of freedom, likewise, are divided into:

(1) Average regression of bacteria on days, independent of changes in moisture—1 degree of freedom.

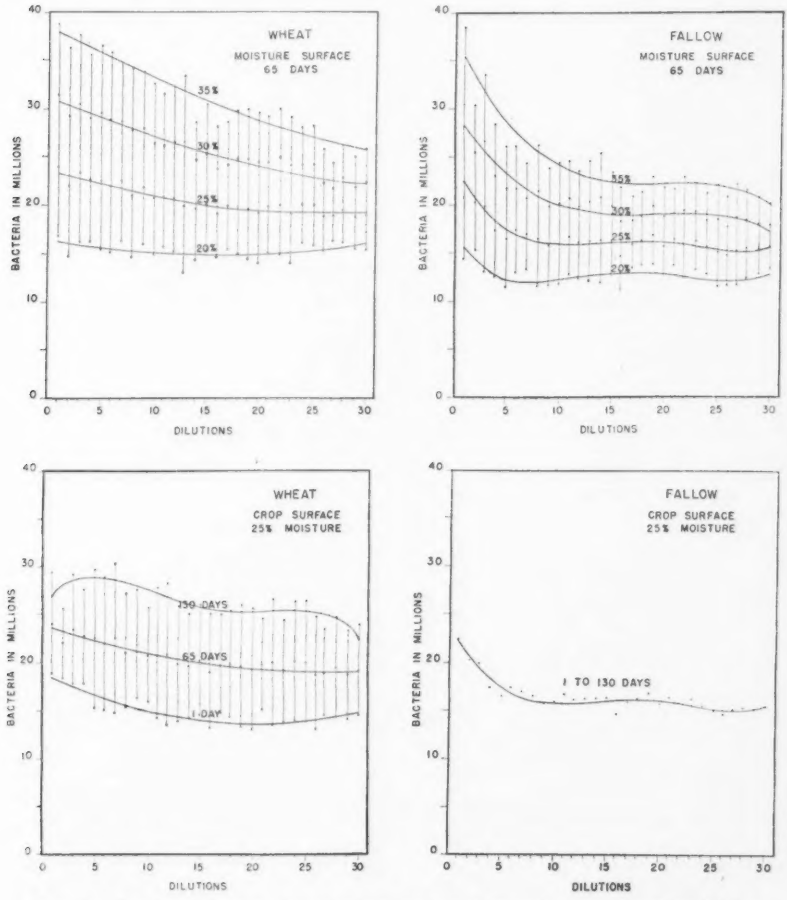


FIG. 3. *Regular differences in estimated numbers of bacteria, as reflected by different dilutions. Covering a range from 20 to 35% moisture at 65 days in each moisture surface, and from 1 to 130 days at 25% moisture in each crop surface.*

(2) Differences between dilution regressions of bacteria on days, independent of changes in moisture—29 degrees of freedom. In this case the 30 dilution regressions are represented, in Fig. 3, by the perpendicular lines on the crop surface for the wheat plot data. Since there is no significant time effect in the fallow plot data, the estimate at 25% moisture from any dilution is the same at 1, 65, or 130 days. Consequently, the time effect at 25% moisture is expressed by a series of 30 points on the crop surface.

(*c*) *Error.* Wheat plot—480 degrees of freedom.
Fallow plot—510 degrees of freedom.

This is the sum of squares for within dilutions not explained by (*a*) and (*b*). Or, it is the sum of squares for the deviations from the regression within Dilution 1 added to that for the deviations within each of the other dilutions. The distribution of these deviations is shown in Fig. 4.

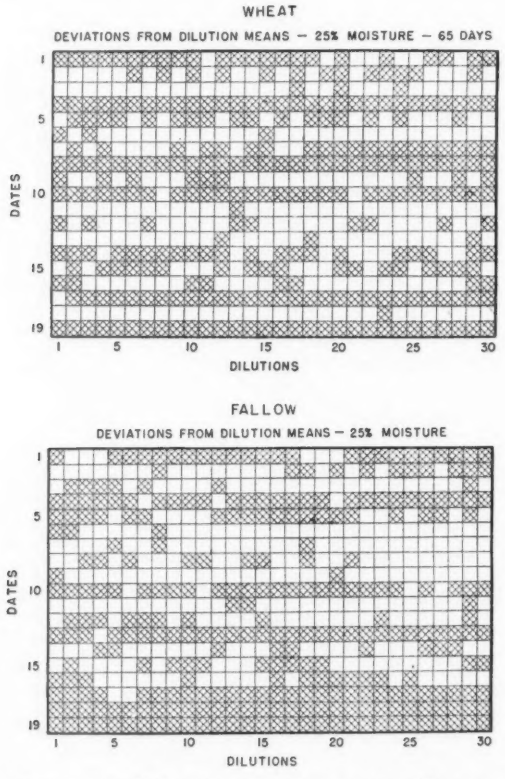


FIG. 4. *Distribution of the 19 deviations from the regressions of bacteria on moisture and on time within each dilution, shown for the 30 dilutions,—that portion of the variation indicated in Fig. 2 not explained by the vertical regression lines in Fig. 3. Negative deviations—shaded squares, and positive deviations—blanks.*

The analysis of variance and tests of significance for each plot are presented as follows:

Source of variance	Sum of squares	D.f.	Variance	*F*
Wheat plot				
Total	25153.7359	569		
Between dilutions	1142.1812	29		
(*a*) Curve	927.1734	2	463.5867	58.2154**
(*b*) Deviations from curve	215.0078	27	7.9633	
Within dilutions	24011.5647	540		
(*a*) *rBM.D*				
(1) Average regression	8174.1369	1	8174.1369	404.2840**
(2) Between dilution regressions	587.6546	29	20.2640	1.0022
(*b*) *rBD.M*				
(1) Average regression	5379.3829	1	5379.3829	266.0585**
(2) Between dilution regressions	165.3544	29	5.7019	0.2820
(*c*) Error	9705.0359	480	20.2188	
Fallow plot				
Total	13483.4407	569		
Between dilutions	1527.4556	29		
(*a*) Curve	1403.9841	4	350.9960	65.3831**
(*b*) Deviations from curve	123.4715	25	5.3683	
Within dilutions	11955.9851	540		
(*a*) *rBM*				
(1) Average regression	1215.4454	1	1215.4454	95.7994**
(2) Between dilution regressions	4269.9445	29	147.2395	11.6052**
(*b*) Error	6470.5952	510	12.6874	

** *Above the 1% level of significance.*

Certain points in this analysis appear worthy of comment. They are considered in order.

1. The curve at 25% moisture and 65 days which expresses the relationship between dilution estimates and dilution numbers is highly significant in the data for each plot. From this it is apparent that some factor associated with different numbers of colonies on plates produces systematic and serious variation in the data. Consequently, if the number of colonies per plate differs, estimates made under constant conditions by the plate count method are not always directly comparable. Of course, such estimates provide valid information when the data are subjected to appropriate statistical treatment.

2. In the fallow plot data there is wide variation among dilutions in the rate of change in numbers of bacteria per unit change in moisture, as is shown by the highly significant *F* value for between dilution regressions of bacteria

on moisture. This variation among dilutions in response to changes in moisture, independent of time, is much less in the wheat plot data, as is indicated by an F value of 1.0022 for between dilution regressions of bacteria on moisture, with days constant, while the variation among dilutions in response to time, independent of moisture, is very small. The F value for between dilution regressions of bacteria on moisture in each plot merely indicates variation among dilutions that might be distributed at random among the 30 dilutions. That this is not the case can be observed in the fallow and wheat plot moisture surfaces shown in Fig. 3. The individual dilution regression lines gradually become shorter from Dilution 1 to Dilution 30. That is, in each plot the population response to moisture decreases regularly as the number of colonies on plates increases, whereas the response to time in the wheat plot is apparently about the same in all dilutions even though the number of colonies on plates differs widely.

3. There is striking similarity in the analysis of variance data for the two plots in that highly significant responses to changes in environment are demonstrated in both. The populations, however, apparently respond differently to these changes. An attempt to test the significance of these differences between plots is not considered since lack of biological information on changing soil populations precludes the possibility of intelligent interpretation at this time.

4. Bacterial responses to changes in environment explain a large portion of the total sum of squares for bacteria in each plot. Since these responses represent a relatively small number of degrees of freedom the error variance is reduced. The following data show this in a striking manner.

Source of variance	Sum of squares	D.f.	Variance	F
Fallow plot				
Total (from Fig. 1)	13483.4413	569	23.6967	
Explained (Fig. 3)	6889.3746	34	202.6287	16.4399**
Unexplained (Fig. 5)	6594.0667	535	12.3254	
Wheat plot				
Total (from Fig. 1)	25153.7459	569	44.0269	
Explained (Fig. 3)	15233.7022	62	245.7049	12.5576**
Unexplained (Fig. 5)	9920.0437	507	19.5662	

** *Above the 1% level of significance.*

Thus, when the population responses to moisture and time as reflected by different dilutions are removed from the variation in the raw data the estimates vary as shown in Fig. 5. Or expressed in another way, the residual variation in the fallow plot data gives a variance of only 52.01% of that for

the variation in the original data. Similarly, in the wheat plot data the variance is reduced to 44.26%.

It is readily apparent therefore that, even if information on the responses of the population to changes in the factors discussed is not desired, their consideration would reduce the error used to test other factors by about 50% and would thereby increase the sensitivity and potential usefulness of an experiment based on plate count data.

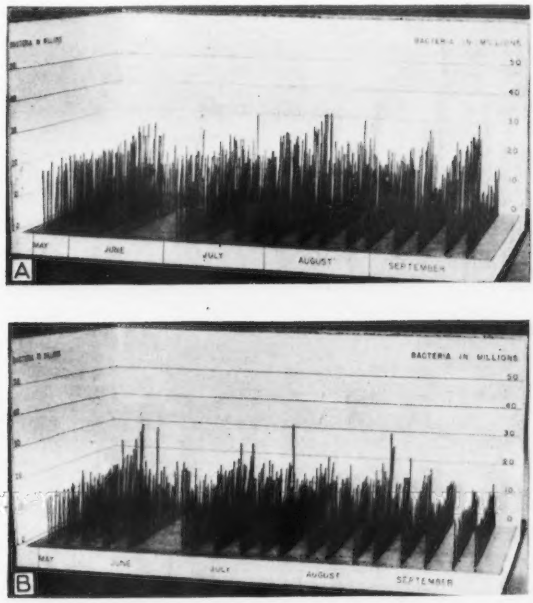


FIG. 5. *Residual variation in numbers of bacteria—that portion of the variation shown in Fig. 1 not explained by differences in moisture, in dates of sampling, and in dilutions. May 21 to Sept. 27—left to right; Dilutions 30 to 1—front to back; bacteria in millions as vertical lines. A—wheat plot, B—fallow plot.*

Acknowledgments

The authors are pleased to express thanks to Dr. C. H. Goulden, Senior Agricultural Scientist, Dominion Rust Research Laboratory, Winnipeg, Man. and Honorary Lecturer in Statistics, The University of Manitoba, for assistance in the statistical part of this study, and to Mr. A. M. Brown, Assistant Plant Pathologist, of the Dominion Rust Research Laboratory, for photographing the illustrations.

References

1. JAMES, N. and SUTHERLAND, M. L. Can. J. Research, C, 18 : 347-356. 1940.
2. JAMES, N. and SUTHERLAND, M. L. Can. J. Research, C, 18 : 435-443. 1940.
3. JAMES, N. and SUTHERLAND, M. L. Can. J. Research, C, 20 : 435-443. 1942.
4. JAMES, N. and SUTHERLAND, M. L. Can. J. Research, C, 20 : 444-456. 1942.

THE ARTIFICIAL SYNTHESIS OF A 42-CHROMOSOME SPECIES RESEMBLING COMMON WHEAT[1]

By W. P. Thompson[2], E. J. Britten[3], and Jean C. Harding[4]

Abstract

An attempt has been made to repeat the hypothetical events in the origin of common wheat. *Triticum turgidum* ($n = 14$), a species belonging to the emmer section of the genus, was crossed with *Aegilops speltoides* ($n = 7$), and doubling of the chromosomes in F_1 was induced by the colchicine method. Four generations of the resulting amphidiploid have been raised. The new synthetic type has the chromosome number ($n = 21$) of the group of species to which bread-wheat (*Triticum vulgare*) belongs. It has most but not all of the distinguishing characteristics of the vulgare group and is fairly fertile. It crosses readily with natural *T. vulgare*; the hybrids are fertile, and the great majority of their chromosomes behave normally at meiosis. It is concluded that the vulgare section of the genus *Triticum* arose in prehistoric times through a similar series of events.

Introduction

The species of wheat fall into three natural taxonomic groups, the einkorn, the emmer, and the bread-wheat series. The latter include *Triticum vulgare*, the common bread-wheat, *T. spelta*, and *T. compactum* (hereafter for convenience called collectively the vulgare group). The emmers include several species of which perhaps the best known are *T. durum* and *T. turgidum*, the macaroni and rivet wheats. According to a widely accepted theory the vulgare group originated in prehistoric times through the natural crossing of a member of the emmer group with a member of the related genus *Aegilops*. This theory was proposed by Percival (6) on purely taxonomic grounds. Most of the characters that distinguish the vulgare from the emmer series are found in *Aegilops*.

Subsequent analysis of the chromosome situation in the genera *Triticum* and *Aegilops* and their hybrids supported the theory. Both genera have diploid, tetraploid, and hexaploid species, with seven as the basic chromosome number. All emmers are tetraploid ($n = 14$) and all vulgares, hexaploid ($n = 21$). In hybrids between members of the two groups all 14 emmer chromosomes pair normally with 14 vulgare chromosomes. From this it is concluded that two of the three sets of seven chromosomes present in vulgares have come from an emmer ancestor. That the remaining set of seven has come from *Aegilops* is indicated by the fact that in hybrids between vulgares and certain species of *Aegilops* (e.g. *A. cylindrica*) there are seven closely mated pairs, whereas in hybrids between emmers and the same species of *Aegilops*, there is no pairing. If different sets of seven chromosomes be designated

[1] *Manuscript received December 28, 1942.*
Contribution from the Department of Biology, University of Saskatchewan, Saskatoon, Sask.

[2] *Professor of Biology.*
[3] *Research Assistant, Department of Genetics, University of Wisconsin, Madison, Wis.*
[4] *Holder of a Bursary under the National Research Council of Canada.*

A, *B*, *C*, the whole situation may be represented as follows: all emmer wheats contain *A* and *B*; all vulgares *A*, *B*, and *C*; certain *Aegilops* species contain *C* (and may contain *D* or *E*).

In the hypothetical cross by which vulgare wheats are believed to have been produced, Sets *A* and *B* were contributed by the emmer parent and Set *C* by *Aegilops*. The presumably sterile F_1 would be *ABC*. Doubling of all chromosomes would restore fertility and would produce the vulgare somatic condition *A A B B C C*. It is possible of course that subsequent translocations and other alterations would modify the original chromosome complements and destroy the distinctness of the original sets. It should be mentioned that the two species of *Aegilops* that Percival suggested as probable ancestors, *A. cylindrica* and *A. ovata*, do not fit into this scheme as they have 14 haploid chromosomes; but there are other species of *Aegilops* that have only seven.

An artificial repetition of these hypothetical prehistoric events in the origin of common wheat became possible through the discovery of the colchicine method of causing doubling of chromosomes. A test of the theory could therefore be made. The obvious procedure was to cross an emmer with a species of *Aegilops* that has seven haploid chromosomes and some vulgare characters, and double the chromosomes in the hybrid through the use of colchicine. If the theory is correct the resulting amphidiploid should be fertile and should resemble the vulgare group. The present paper describes the results of such an experiment.

Other amphidiploid hybrids between *Triticum* and *Aegilops* have been reported but they had 56 chromosomes (7, 11) or 28 (8), not 42 as in vulgare wheats.

A preliminary report of the early stages of this experiment has been published (1).

Materials and Methods

The emmer species chosen for crossing was *T. turgidum* ($n = 14$) and the *Aegilops* was *A. speltoides* ($n = 7$). After the amphidiploid was produced four generations were grown. F_2 and F_3 plants were crossed with *T. vulgare* var. Marquis and *T. spelta* in order to test the possible relationship of the synthetic type to natural members of the vulgare series.

Colchicine Treatment

Members of the Gramineae do not lend themselves well to the colchicine technique for chromosome doubling. Thorough trial of various suggested methods and modifications of them showed that the following was most successful for our material:

Two days after the seedling emerged 0.2% aqueous solution of colchicine was injected by hypodermic needle well below the end of the sheath in the position in which dissection of similar seedlings showed that the growing point was likely to be located. The solution was forced in until drops appeared at the top of the sheath. This treatment was repeated daily for nearly a

month, being suspended temporarily only when the plants showed signs of severe injury. Each new stem as it appeared was treated similarly.

Cytological Methods

The acetocarmine technique was used throughout; all slides that were carefully studied had been made permanent. With freshly fixed material this technique was entirely satisfactory, but as the preserved material stood in alcohol it became more and more difficult to spread the chromosomes satisfactorily even with excessive pressure on the cover glass. This difficulty was overcome by preserving the material in a mixture of equal parts of glycerine and 70% alcohol. Such material spread easily and its staining qualities were not impaired. It tended to fragment but this was overcome by adjusting the percentage of glycerine or by returning the material to alcohol a short time before use.

The Undoubled First Generation

Untreated F_1 plants are always completely sterile on both the male and female sides.

According to Jenkins (3) four to 10 (usually seven) bivalents appear at meiosis and are attached only end-to-end at metaphase. The remaining one to 13 (making 21 chromosomes in all) appear as univalents and behave as usual in wheat hybrids. Jenkins reported that several cells were observed in which a bivalent and univalent were joined end-to-end.

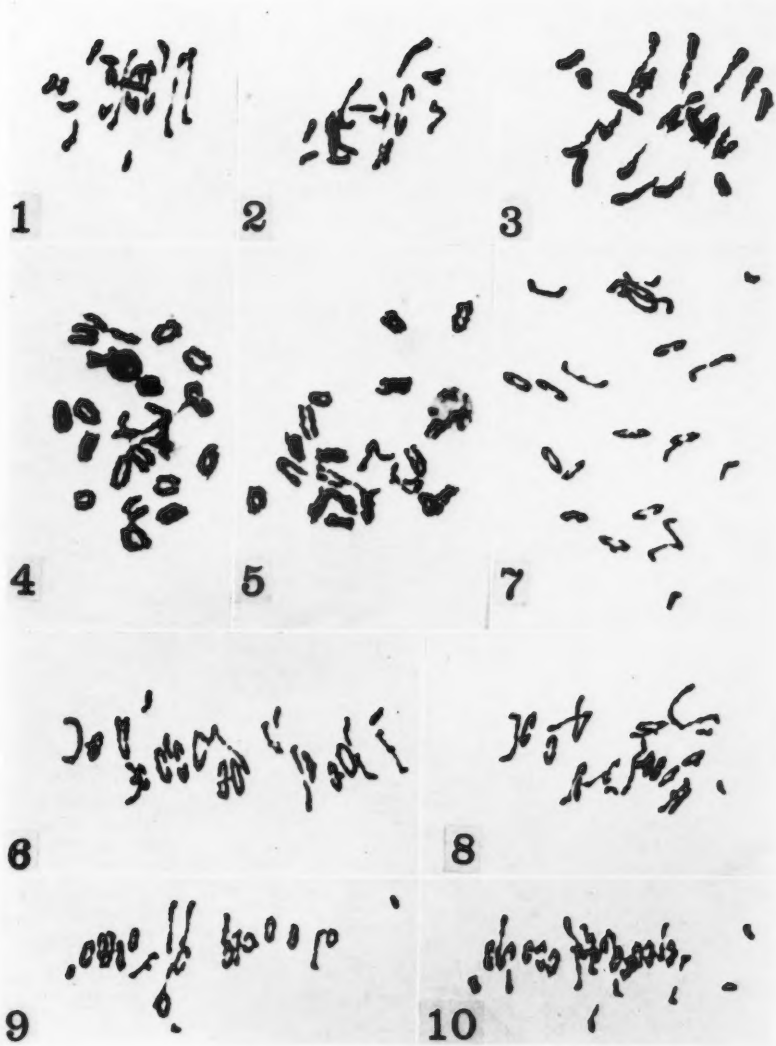
Because of certain features in the behaviour of the amphidiploid (to be reported in another section), it became necessary to make a careful re-examination of the sterile F_1. We find that associations of three occur in more than 80% of the cells; usually only one or two, but sometimes three, are found. The number of bivalents is considerably lower in the material studied than Jenkins reports, and various numbers of univalents (not merely the odd numbers reported by him) occur. Figs. 1 to 3 show cells with different combinations of typical univalents, bivalents, and associations of three. Table I gives their frequencies in 57 cells that were completely analysed.

The pairing is usually very loose. Less than 20% of the bivalents observed at metaphase formed closed rings.

TABLE I

FREQUENCY OF POLLEN MOTHER CELLS IN UNTREATED F_1 WITH VARIOUS NUMBERS OF STATED ASSOCIATIONS OF CHROMOSOMES

Association	Number													
	0	1	2	3	4	5	6	7	8	9	10	11	15	16
Univalent			1	4	6	6	5	11	7	5	6	4	1	1
Bivalent		1	2	2	16	15	11	9	1					
Trivalent	10	24	19	4										



EXPLANATION OF FIGURES

All figures are photomicrographs of first meiotic divisions at a magnification of approximately 650.

FIGS. 1 TO 3. *Sterile (undoubled) F_1 of T. turgidum × A. speltoides.* FIG. 1. *Metaphase showing one trivalent (third from right end), five bivalents, and eight univalents.* FIG. 2. *Six II, nine I.* FIG. 3. *Three III at right, all V-shaped, three II, six I.*

FIGS. 4 TO 6. *Amphidiploid.* FIG. 4. *Diakinesis showing one IV in the centre and 19 II.* FIG. 5. *Diakinesis showing two III and 18 II.* FIG. 6. *Metaphase of 41-chromosome plant; one IV near centre, two V-shaped III (third from left and near centre), 14 II, three I.*

FIGS. 7 AND 8. *Amphidiploid × T. vulgare.* FIG. 7. *One IV, one III, 15 II, four I.* FIG. 8. *Two IV, two III, 12 II, four I.*

FIGS. 9 AND 10. *Amphidiploid × T. spelta.* FIG. 9. *Nineteen II, three I.* FIG. 10. *One zigzag IV, near left, 17 II, four I.*

The associations of three almost always formed *V*-shaped configurations at metaphase (see Fig. 3). Consequently the two terminal chromosomes go to one pole and the middle chromosome to the other. This regularly alternate segregation of associations of three is of particular interest in view of the observations (9, 10) that alternate segregation is the rule in translocation complexes of four and six chromosomes in wheat.

The Doubled First Generation

Two F_1 plants were grown under very favourable conditions and treated systematically with colchicine according to the method already described. One produced 70 spikes, of which 63 were completely sterile and seven quite fertile. The other produced 72 fertile and eight sterile spikes. Each spike was either completely sterile or reasonably fertile. The 15 fertile spikes bore 206 seeds. Work on their offspring showed that these seeds had the doubled chromosome number. Presumably the 15 fertile spikes were produced from growing points in which doubling of the chromosomes had occurred as a result of the colchicine treatment.

Pollen had been collected from two of the spikes which later set good seed. In one of these 81% of the pollen grains and in the other 65% appeared to be normal. In other spikes examined every pollen grain was empty, as in untreated plants.

Second to Fourth Amphidiploid Generations

Chromosomes

Table II gives the chromosome numbers of 53 unselected amphidiploid plants. It will be observed that the great majority have the same diploid number as vulgare wheats ($2n = 42$) and that nearly all the remainder depart by only one or two from that number. Some plants with abnormal numbers are to be expected in view of the chromosome behaviour to be described below.

TABLE II

Frequency of amphidiploid plants with various numbers of chromosomes

Generation	Chromosome number						
	34	39	40	41	42	43	44
F_2			3	3	16		1
F_3	1		2	1	16		
F_4		1	2	2	5		
Total	1	1	7	6	37		1

A typical 42-chromosome amphidiploid plant in any generation has some pollen mother cells with 21 regular pairs of chromosomes, but the great majority of cells show a few unpaired chromosomes as well as associations of

three or four. For convenience these associations will be referred to as trivalents and quadrivalents, but it is not intended to imply that all the members of any association are fully homologous; they may be the result of translocations.

Figs. 4 and 5 show diakinesis in the amphidiploid. In the former, 19 pairs (two loose) and an association of four (at the centre) may be seen, in the latter, 18 pairs plus two trivalents. In the metaphase photographed in Fig. 6, a quadrivalent, two *V*-shaped trivalents, and three univalents may be seen.

The number of irregular associations varies from cell to cell at the same stage in any plant, from stage to stage, and from plant to plant. Unpaired chromosomes are fewer at diplotene and diakinesis than at metaphase, whereas quadrivalents are more numerous. Apparently some of the associations formed in prophase tend to disjoin before metaphase.

Preparations from 35 plants were surveyed systematically with respect to unpaired chromosomes. In 28 plants there were at least a few cells with all chromosomes paired; in the rest all cells showed some unmated. The average number of unpaired chromosomes per cell was two to four. The maximum number varied from two to 14. It is evident that in typical plants all chromosomes can pair and that nearly all of them usually do pair.

The number of trivalents and quadrivalents also varies. In every plant most meiotic cells show at least one such association; many show one or two of each. Since bivalents and trivalents are present in the undoubled F_1, such associations are to be expected. Table III gives the combinations of uni-,

TABLE III

COMBINATIONS OF CHROMOSOMES IN DIFFERENT CELLS OF THE AMPHIDIPLOID

I	II	III	IV	
	21			(Two cases)
	19		1	(Two cases)
1	19	1		
4	19			
3	18	1		
6	18			
2	17	2		
3	14	1	2	(Two cases)
2	12	4	1	
3	11	3	2	

bi-, tri-, and quadrivalents (I, II, III, IV) that were found in an unselected series of cells. The irregularities result in the production of a small proportion of plants with abnormal chromosome make-up, and these in turn show a larger proportion of irregularities than typical plants do.

The segregation of the trivalents and quadrivalents is typically alternate. The former usually take the form of a *V* at metaphase (Fig. 6) with two terminal chromosomes going to one pole and the middle one to the other. The quad-

rivalents usually form a zigzag configuration (Figs. 6 and 8) similar to the broken figure of eight in translocation complexes (9). The unbroken figure of eight is rarely seen.

Larger associations consisting of five or six chromosomes have been seen but they are rare.

FERTILITY

Pollen.—Table IV shows the percentage of normal pollen grains in 90 unselected plants. It will be observed that the great majority of plants have from 75 to 95% good pollen. In preparing this table grains that had normal cytoplasmic content but less than the usual amount of starch were counted as good. If they are regarded as infertile the percentage of good pollen would be reduced in each plant by about 10. That there is an abundance of fertile pollen is indicated also by the good production of seed following selfing and by the fact that the amphidiploid can readily be crossed as male with *T. vulgare.*

TABLE IV

FREQUENCY OF AMPHIDIPLOID PLANTS WITH DIFFERENT PERCENTAGES OF GOOD POLLEN

Generation	Percent good pollen					
	85–100	85–94	75–84	65–74	55–64	Below 55
F_2	2	18	8	2		
F_3		20	16	5	1	1
F_4	1	2	4			

Seeds.—The number of seeds per spike borne on 51 unselected F_3 and F_4 plants is shown in Table V. This represents a fair degree of fertility, though considerably lower than that of pure wheat. Three of the 51 plants examined were completely sterile. In view of the chromosome irregularities described earlier some sterility is to be expected. The higher degrees of sterility were found in the more irregular plants, particularly those with abnormal numbers.

TABLE V

FREQUENCY OF PLANTS WITH VARIOUS NUMBERS OF SEEDS PER SPIKE

Seeds per spike	0	1–4	5–9	10–14	15–19	20 or more
Number of plants	3	14	15	9	6	4

GENETIC CHARACTERS

Since this work is concerned with the origin of common wheat and since it is clear from cytological evidence that one of the ancestors must have been an emmer, consideration will first be given to the characters that distinguish

vulgares from emmers. (The term "vulgares" is here used to designate all the species of the vulgare group, including *T. spelta*.)

The nature and distribution of the hairs on the leaf constitute an important diagnostic feature. In vulgare and *Aegilops* there is a single row of long hairs on the summit of each ridge and shorter ones on the sides; in emmers they are of uniform length. The new type fully resembles vulgares.

The stems of vulgares are larger than those of emmers which are in turn larger than those of *A. speltoides* (although other species of *Aegilops* resemble vulgares in this respect). Those of the new type lie within the range of emmers (1.3 to 1.6 mm. at a point 2 cm. below the spike). In vulgares and *Aegilops* the stems are hollow with thin walls, in emmers solid (at least above; they may have a very small cavity and thick walls lower down). The new type has hollow stems throughout but the walls are somewhat thicker than those of vulgares.

The spike of emmers is dense, of *T. vulgare* lax, and of *T. spelta* and *Aegilops* very lax; the amphidiploid is like *spelta* in this respect. The collar at the base of the spike is open as in *vulgares*.

The bearded varieties of *vulgare* have shorter beards than emmers. In this respect, too, the amphidiploid is near *vulgares*.

The important characters of the empty glume are almost entirely vulgare-like including (1) the rounded, *U*-shaped back, (2) the wide, truncated end (shoulder), and (3) the weakly developed keel. These are in contrast to the *V*-shaped back, the narrow sloping end, and the prominent keel of all emmers. The general shape of the glume in lateral view is similar to that of *T. spelta*, a long and fairly wide rectangle.

The rachis of the new type is very fragile so that the spike shatters very easily. This is not a diagnostic feature since both fragile and non-fragile species occur in both emmer and vulgare groups as well as in *Aegilops*. But the mode of disarticulation is characteristic. In fragile emmers the break occurs at the nodes, whereas in *T. spelta*, the fragile member of the vulgare series, it occurs just below the spikelets. The amphidiploid resembles the emmers in this respect. But the matter is probably not of much significance in relation to the origin of non-fragile *vulgare* since, as stated, there are non-fragile species of emmer (including the *turgidum* parent of the amphidiploid) and of *Aegilops*.

The slender segments of the rachis with their tops much wider than their base are definitely emmer-like.

While there is much variation in different species of *Triticum* with respect to the hairs on the rachis, the coarse lateral fringe with large marginal tufts at the top of each segment are typical of *T. spelta*.

The shape of the cross section of the seed is like that of vulgare, but this should not, perhaps, be considered an independent character since it may be determined by the shape of the glume.

In respect to nine of the characters in this list the new type is like members of the vulgare series, in one it is near vulgare, in three it is emmer-like. Within the vulgare group it is in several respects nearer to *T. spelta* than to *T. vulgare.*

Some characters which do not serve to distinguish vulgares from emmers are as follows in the amphidiploid. The spike is nearly square in section, like that of *T. spelta* and some emmers, whereas in *T. vulgare* the one-ranked side is wider and in some emmers the two-ranked side. The tooth at the apex of the empty glume is short and blunt as in *T. spelta* and most varieties of *T. vulgare*: in emmers it is long and sharp; but some bearded varieties of vulgare also have sharp teeth. The glumes adhere tightly to the seed. The seed itself is long and tapers towards both ends. It has a tuft of hairs at the apex which are longer and more numerous than those of most emmers, but not so long or numerous as those of vulgares.

The amount of variation in genetic characters is surprisingly small in view of the meiotic irregularities.

Crosses of Amphidiploid with Species of the Vulgare Group

Several F_2 and F_3 plants of the new synthetic type were crossed with *T. vulgare* and *T. spelta*. The number of seeds set following the cross-pollination was almost as high as in varietal crosses in wheat. This was true whether the amphidiploid was the male or the female parent. The crossed seeds were, however, badly shrivelled and germinated poorly.

Five F_1 plants of the cross with *T. spelta* and three of the cross with *T. vulgare* have been grown. With one exception they were vigorous, healthy plants. It was difficult to estimate the percentage of fertile pollen because all gradations occurred from apparently normal grains, through ones with somewhat reduced starch content, with no starch, with reduced cytoplasm, to completely empty grains. From 40 to 70% of the pollen grains appeared perfectly normal both in cytoplasmic and starch content. All the field-grown hybrid plants were fertile. The hybrids with spelt averaged four seeds per spike and those with vulgare, seven.

The ease of crossing, the vigour of F_1 and their considerable degree of fertility on both male and female sides, all indicate a close relationship between the synthetic and the natural types.

Five of the hybrid plants had 42 chromosomes and the remaining three had 41, 40, and 39. These numbers are in accordance with expectations from the meiotic behaviour of the amphidiploid and the contribution of 21 by the vulgare parent.

Fig. 7 is a photograph of a typical first meiotic division in a hybrid (41 chromosomes) between the amphidiploid and *T. vulgare.* It will be seen that there are three unpaired chromosomes, two trivalents, a quadrivalent, and 14 bivalents, most of which are closely paired. Fig. 8 is a metaphase from a 42-chromosome plant of the same cross. Figs. 9 and 10 show typical cells at metaphase in the hybrid between the amphidiploid and *T. spelta.*

The number of unpaired chromosomes and of associations of two, three, and four in each of a considerable number of cells is given in Table VI. These cells were selected only to the extent that they were the clearest for complete analysis. The number of unpaired chromosomes varies from one to 10. It is always much lower than it would be if the natural and synthetic types were not closely related.

TABLE VI

CHROMOSOME PAIRING IN DIFFERENT CELLS OF 42-CHROMOSOME HYBRID BETWEEN THE AMPHIDIPLOID AND *T. spelta* (UPPER SECTION) AND IN 41-CHROMOSOME HYBRID BETWEEN THE AMPHIDIPLOID × *T. vulgare* (LOWER SECTION)

I	II	III	IV	
2	20			(Two cases)
2	18		1	
3	18	1		
6	18			
1	17	1	1	
1	16	3		
3	16	1	1	(Two cases)
8	14	2		
7	12	1	2	
3	19			(Two cases)
5	18			
4	17	1		(Two cases)
6	16	1		
9	16			
5	15	1	1	
3	14	2	1	
10	14	1		
8	13	1	1	
4	13	2	1	
9	13	2		
10	12	1	1	

Discussion

All the species of *Triticum* and related genera must have descended from a seven-chromosome ancestor. Several types must have differentiated without change of chromosome number. One of these types was an einkorn (like *T. monococcum*); a second, now unknown, subsequently crossed with an einkorn to produce, after chromosome doubling, the progenitor of the emmer series ($n = 14$). A third seven-chromosome type was an *Aegilops*. According to the present hypothesis it crossed with an emmer to produce, after another doubling of the chromosomes, the progenitor of the vulgare series ($n = 21$).

The origin of common bread-wheat therefore involves two problems: (1) the origin of the progenitor of the whole vulgare series of species, (2) the derivation of *T. vulgare* itself within that series. We are here concerned with the first of these problems—whether the present work confirms the hypothesis outlined, and whether the hypothetical prehistoric events have been artificially duplicated.

The new synthetic type has the same chromosome number ($2n = 42$) as the vulgare series and they are the only wheats with that number. It is true that a small proportion of plants with abnormal numbers near 42 also occur. But these are largely sterile and there is every reason to expect that the typical number 42 will persist.

The meiotic behaviour of the chromosomes is nearly regular. The occurrence of a small proportion of associations of three or four, which frequently disjoin before metaphase into univalents and bivalents, is to be expected in view of the occurrence of some loose pairing in the sterile F_1. This F_1 pairing may be in part between chromosomes of *T. turgidum* which are ordinarily regarded as non-homologous. Kihara and Nishiyama (4) reported evidence that in the absence of their normal mates some emmer chromosomes may pair together abnormally. But the amount of pairing in some F_1 cells and the occurrence of trivalents indicate that some *Aegilops* chromosomes are involved. Presumably the *Aegilops* and *Triticum* chromosomes have retained from their common ancestor sufficient homology to make occasional loose pairing possible. In the course of time translocations and other chromosome alterations have destroyed the distinctness of the sets of seven. When, through doubling, each chromosome acquires a proper mate, the pairing is almost always in pairs to form normal bivalents, but the same relationships that cause loose pairing in F_1 may cause occasional associations of three or four in the doubled F_2. The chromosome behaviour therefore is that of a nearly, but not completely "good" species.

There is sufficient chromosome irregularity to cause the production of occasional sterile or partially sterile plants, but many individuals of the new type show nearly normal fertility on both the male and female sides. Conditions respecting fertility are therefore no bar to regarding the synthetic type as an essential though not complete duplication of the natural type of vulgares.

In the great majority of its genetic characters the new type is a member of the vulgare series. This is particularly true of those characters that are regarded by taxonomists (6) as most important in distinguishing vulgares from emmers. But in a few respects it resembles emmers and cannot therefore be regarded as a complete artificial duplication of vulgares.

The best test of the relationship between the artificial and natural types is the results of crossing them. It has been shown that the synthetic type crosses readily in either direction with both *T. vulgare* and *T. spelta* and that the hybrids are vigorous and partially fertile. The poor quality of the seeds indicates, however, that the two are not identical. This estimate of their relationship is confirmed by the meiotic behaviour of the chromosomes in the hybrids. Unless the *Aegilops* chromosomes are able to pair normally with the seven from vulgare (Set *C*) which cannot pair with those of emmers, at least 14 unmated chromosomes should be found regularly. (The remaining 14 from *vulgare* would be expected to pair with the 14 from *turgidum*, as they do in hybrids made directly between *vulgare* and *turgidum*.) The normal pairing of the great majority of the chromosomes therefore indicates close

relationship. But the occurrence of a few unpaired ones and associations of three or four, although they are only slightly more numerous than in the amphidiploid itself, indicates a lack of complete identity.

From a consideration of all these points, the conclusion may be drawn that the natural vulgare type has been artificially synthesized in most essential respects. It has not, however, been completely duplicated. Changes in both the *Aegilops* and emmer parent as well as in the vulgare type may have occurred subsequent to the original prehistoric cross. Perhaps the use of some other species of emmer or *Aegilops* would more completely duplicate living vulgares. Or perhaps the actual parental species are now extinct.

Within the vulgare section of the genus the origin of *T. vulgare* (bread-wheat) involved no change in chromosome number but probably involved a structural alteration of one or more chromosomes. Speltoid mutations are of frequent occurrence in *T. vulgare*. According to several workers (2, 5, 12) many of these mutations and perhaps all of them are due to deficiencies of part or all of a chromosome. Evidently the change within the vulgare section that differentiated *T. vulgare* and *T. spelta* was not very great. In this connection it is important to note that the synthetic type resembles *T. spelta* more than *T. vulgare*. This is to be expected since *T. spelta* is the more primitive type.

References

1. Britten, E. J. and Thompson, W. P. Science (n.s.), 93 : 479. 1941.
2. Huskins, C. L. Proc. World's Grain Exhib. Conf., Regina, Canada, 1933, 2 : 45-50. 1935.
3. Jenkins, J. A. Am. J. Botany, 16 : 238-245. 1929.
4. Kihara, H. and Nishiyama, I. Cytologia, 1 : 263-284. 1930.
5. Love, R. M. Abstract. Genetics, 25 : 126. 1940.
6. Percival, J. The wheat plant. Duckworth and Company, London. 1921.
7. Percival, J. Ann. Botany, 50 : 427-436. 1936.
8. Sears, E. R. J. Hered. 30 : 38-43. 1939.
9. Thompson, W. P. and Hutcheson, I. Can. J. Research, C, 20 : 267-281. 1942.
10. Thompson, W. P. and Thompson, M. G. Cytologia, Fujii Jubilee Volume, Pt. 1 : 336-342. 1937.
11. Tschermak, E. Ber. deut. botan. Ges. 47 : 253-261. 1929.
12. Uchikawa, I. Mem. Coll. Agr., Kyoto Imp. Univ. No. 50 : 1-64. Genetical Ser. No. 9. 1941.

A VISUAL FLUOROMETER FOR THE CHEMICAL ESTIMATION OF VITAMIN B_1 IN WHEAT FLOUR[1]

By E. J. Reedman[2] and G. A. Young[3]

Abstract

A simple, inexpensive, visual fluorometer has been constructed for measuring the fluorescence of thiochrome converted from extracted thiamin by oxidation. The resulting thiamin estimations on wheat flour compare well with those obtained by other methods, and can be read to within 0.2 μg. per gm. Details of the rapid chemical method are given.

Introduction

The thiochrome method of Jansen (4) has been widely used for the determination of vitamin B_1. Modifications by Hennessy and Cerecedo (3), Pyke (6), and others, have been used extensively in conjunction with photoelectric fluorometers for measuring the fluorescence of thiochrome converted from extracted thiamin by oxidation. This paper describes a simple, inexpensive, visual fluorometer designed to replace the photoelectric fluorometer in the thiochrome method. Existing visual procedures require the addition of standard solutions as a measuring device (5), whereas the instrument herein described may be calibrated to read directly.

Method

Since wheat contains little or no combined vitamin B_1 (1), enzymic digestion to liberate thiamin from phosphoric esters is not necessary, and thiamin extractions with dilute acid have therefore been found adequate (5). Details of the modified rapid method follow.

Extraction

Twenty grams of the flour to be assayed were placed in a 250 ml. centrifuge tube, 100 ml. of 1% hydrochloric acid was added, the tube stoppered and placed on an extraction wheel, and the sample rotated at 30 r.p.m. for 30 min. The tube was then removed from the wheel and centrifuged for 10 to 15 min. at 2000 r.p.m. to obtain a clear supernatant extract.

Certain observations can be made regarding the details of the extraction process, i.e., the 30 min. extraction period was chosen because it was found to give maximum values with the weight of sample, strength of acid, and speed of rotation used; aliquots of the extract may be taken for centrifuging where no apparatus is available for the 250 ml. size tube.

[1] *Manuscript received October 26, 1942.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 206 of the Associate Committee on Grain Research of the National Research Council and Dominion Department of Agriculture, and as N.R.C. No. 1109.

[2] *Biochemist, Food Investigations.*

[3] *Laboratory Assistant.*

Oxidation

Two ml. of the extract were mixed with two ml. of methyl alcohol in a 25 ml. glass-stoppered cylinder, the alcohol being added first. One ml. of 30% sodium hydroxide and one ml. of 1% potassium ferricyanide were added with constant agitation produced by a stream of nitrogen. Bubbling was continued for one minute after the addition of ferricyanide.

Preparation for Measurement

The thiochrome formed was immediately extracted by adding 12 ml. of isobutanol to the cylinder and shaking for one minute. (An emulsion may form, which can be broken up by centrifuging.) The isobutanol layer was decanted or siphoned off, 10 ml. placed in a test tube, and 1 ml. of methyl alcohol added to clarify. The sample was then ready for measurement.

Readings made in a photoelectric fluorometer require a blank, made by treating a 2 ml. aliquot of extract in exactly the same manner as the aliquot taken for assay, except that potassium ferricyanide is omitted. The blank value is subtracted from the thiamin content as read from the calibration curve for the instrument. Readings made in a visual fluorometer do not require a blank.

Certain additional precautions were found to be necessary in the method described. All corks or stoppers were covered with tin-foil, and all glassware freed from extraneous fluorescent materials by exceptionally thorough washing. Fluorescence in the isobutanol itself may be eliminated almost entirely by redistillation. The order of addition of reagents has been found to influence the results (2).

Test of the Method

Samples of two flours, containing high and low natural contents of vitamin B_1 respectively, were assayed biologically by the rat curative method. Analysis of these flours by the rapid chemical method, using the photoelectric fluorometer, gave essentially the same results as the biological assay (Table I). Three mixtures of the high and low vitamin flours were also analysed. These findings indicate that the rapid extraction method gives results comparable to biological values in flours containing a fairly wide range of vitamin B_1 as natural thiamin.

Instrument

Fig. 1 illustrates a visual fluorometer requiring minimum construction for this type of instrument. It may be calibrated to read directly the vitamin B_1 content of the flour on assay in micrograms per gram, International Units per pound, or other convenient unit.

Description

This instrument uses the General Electric *B-H*$_4$ mercury vapour lamp, which is similar to the *H*-4 type of mercury bulb used in photoelectric fluorometers, except for a thick red-purple glass casing. Only ultra-violet light

TABLE I

VITAMIN B_1 ASSAYS IN CALIBRATION SERIES

Sample No.	Composition	Biological assay, μg./g.	Rapid chemical assay, μg./g.
1	High vitamin flour (no synthetic addition)	4.29	4.32
2	High vitamin flour, 75% Low vitamin flour, 25%	3.36 (Calculated)	3.27
3	High vitamin flour, 50% Low vitamin flour, 50%	2.44 (Calculated)	2.60
4	High vitamin flour, 25% Low vitamin flour, 75%	1.52 (Calculated)	1.57
5	Low vitamin flour	0.60	0.60

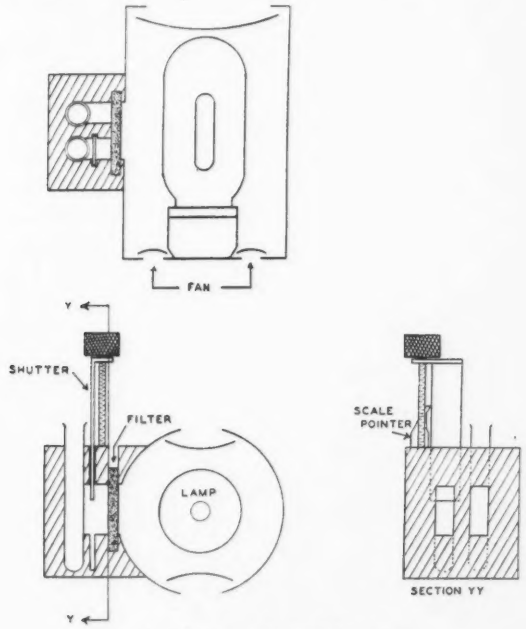


FIG. 1. *Diagram of visual fluorometer.*

emanates from the lamp, and separate filters to eliminate visible light are thus unnecessary. An extra filter (Corning No. 586) may be placed as shown.

The remaining parts consist of a ventilated housing and admedium socket for the lamp, a transformer designed for use with the *B-H*$_4$ type lamp, and a

test tube block mounted as shown. The lamp is cooled by means of a small fan. The mounting of the admedium socket is adjustable, so that the lamp may be centred on the block containing the test solutions. Identical irradition of standard and unknown may be obtained by matching the tubes used (Monax 100 mm. × 13 mm.), and by having openings of the same size behind them, as shown in Section *YY*, Fig. 1. A flat adjustable shutter is provided for the right hand or standard tube, and is used to adjust the amount of irradiation on the standard in matching standard and unknown solutions of thiochrome.

Calibration and Use

Before the instrument can be used it is necessary to calibrate the shutter for the range of vitamin B_1 likely to be encountered in the assayed material. The calibration series may consist of either a standard flour of the type fortified in production, to which definite amounts of synthetic thiamin have been added, or a series of flours containing graded amounts of natural thiamin covering the necessary range.

A series of five flours of known vitamin B_1 content were used in calibration (Table I), the flour with the highest thiamin content being taken as the standard. Isobutanol extracts for two separate samples were prepared by the method described, placed in the fluorometer block and read by looking directly down on the tubes and adjusting the shutter until they matched. The tubes matched with the shutter full open, and this point was marked on the shutter as the thiamin content of the flour in micrograms per gram.

A sample of the flour of next highest thiamin content was then taken for comparison with the standard flour, separate extracts prepared and aliquots oxidized. The shutter was turned down on the standard thiochrome solution until the tubes matched in fluorescence, and this point was marked on the shutter as the thiamin content of the second sample. Similarly, by taking standard and calibration flour and preparing extracts of thiochrome in isobutanol in exactly the same manner, other calibration points were marked on the shutter.

When it is only necessary to ascertain if a given unknown sample is of the required level of fortification, the calibration series should include a sample of fortified flour containing vitamin B_1 in known amount equal to that required. This flour may then be used for direct comparisons.

Extracts of flour with 1% hydrochloric acid have been found to be stable for seven days if protected from direct light (Table II). In general use, therefore, an extract of the standard flour (that of highest thiamin content in the calibration series) may be prepared for each set of unknown samples assayed, and for each aliquot of extract from an assay sample a similar aliquot of standard extract may be taken for oxidation. This procedure may be used to replace that of extracting a standard sample for each sample assayed. It is necessary, however, to use a fresh isobutanol solution for each reading, since, although thiochrome in isobutanol is fairly stable in subdued light

TABLE II

STABILITY OF 1% HYDROCHLORIC ACID EXTRACTS

Time interval between extraction and oxidation	First sample μg./g.	Second sample μg./g.
Immediately	2.88	2.85
Days, 1	2.90	3.00
2	2.95	2.85
3	3.00	2.92
7	2.95	2.85

TABLE III

STABILITY OF ISOBUTANOL EXTRACTS

Time interval between oxidation and reading	First sample μg./g.	Second sample μg./g.
Immediately	0.50	0.50
Hr., 2	0.49	0.49
4	0.47	0.47
24	0.49	0.50
96	0.50	0.47

(Table III), it is rapidly destroyed by ultra-violet light when irradiated in the measurement of fluorescence.

Test of the Instrument

Table IV shows comparisons between readings in the visual fluorometer and measurements made with a photoelectric fluorometer (7). Good agreement was obtained throughout, and it was possible to detect differences of 0.2 μg. per gm. in the original flours by visual comparison. This differentiation is held to be sufficient for control purposes.

TABLE IV

COMPARISON OF THIAMIN VALUES OBTAINED WITH PHOTOELECTRIC AND VISUAL FLUOROMETERS

Actual value (based on bio-assay) μg./g.	Photoelectric fluorometer μg./g.	Visual fluorometer μg./g.
6.55	6.51	6.3
5.62	5.50	5.5
4.69	4.67	4.6
4.11	3.90	3.9
2.85	2.70	2.9
1.50	1.35	1.5

Acknowledgment

The biological assays on the flour samples used in this study were conducted by the Health Laboratories, Department of Pensions and National Health, Ottawa.

References

1. BOOTH, R. G. J. Soc. Chem. Ind. 59 : 181T-184T. 1940.
2. HARRIS, L. J. and WANG, Y. L. Biochem. J. 35 : 1050-1067. 1941.
3. HENNESSY, D. J. and CERECEDO, L. R. J. Am. Chem. Soc. 61 : 179-183. 1939.
4. JANSEN, B. C. P. Rec. trav. chim. 55 : 1046-1052. 1936.
5. NICHOLLS, J. R., BOOTH, R. G., KENT-JONES, D. M., AMOS, A. J., and WARD, H. H. Analyst, 67 : 15-17. 1942.
6. PYKE, M. J. Soc. Chem. Ind. 58 : 338T-340T. 1939.
7. WOODCOCK, A. H. Can. J. Research, D, 19 : 253-257. 1941.

Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 21, SEC. D. APRIL, 1943 NUMBER 4

THE CARDIAC OUTPUT OF FOUR FRESHWATER FISH[1]

BY J. S. HART[2]

Abstract

The stroke output of the heart was determined by measurement of the amount of blood in the ventricle at systole and diastole in four species of fish, the bowfin, *Amia calva* L., the common sucker, *Catostomus commersonii* (Lacépède), the carp, *Cyprinus carpio* L., and the catfish, *Ameiurus nebulosus* (Le Sueur).

The stroke output varies with the size of the individual. Smaller individuals of a species, although possessing a smaller absolute output, have a larger output in relation to their size than do larger individuals.

Over the size range at which they can be compared (300 to 600 gm.) the four species differ in their stroke output. The catfish has the highest output and the sucker the lowest. The bowfin and the carp are intermediate in this respect. At 500 gm., the outputs of the catfish, bowfin, carp, and sucker per stroke are respectively 0.26 gm., 0.22 gm., 0.18 gm., and 0.11 gm.

The results of the present investigation were correlated with those of Black (Biol. Bull. 79 : 215-229. 1940); the output of the heart was found to correlate inversely with the effect of carbon dioxide on the blood of the same species, and directly with the affinity of the blood for oxygen. Differences in the circulation may compensate for differences in oxygen transport imposed by the varying effects of carbon dioxide on the blood.

Introduction

Previous work on oxygen transport in fish has been concerned largely with the physico-chemical properties of the blood. The extensive variations in these properties have indicated a great range in the possibilities for oxygen transport in fish. Often specific dissimilarities in properties of the blood have been related to differences in the external environment in which the fish live.

The properties of the blood of particular interest in this connection are the affinity for oxygen and the effect of carbon dioxide on the oxygen affinity. Krogh and Leitch (4) first noted that these properties varied greatly from species to species, a fact later demonstrated by Root (6), Willmer (7), and Black (1). In the four species studied here, the range of possibilities for oxygen transport by virtue of the carbon dioxide effect alone was pointed out by Black (1). He showed that the differences were such that if all other respiratory characteristics remained the same, the carbon dioxide sensitivity of the common sucker blood might enable it to transport about 15 times as

[1] *Manuscript received September 22, 1942.*

Contribution from the Ontario Fisheries Research Laboratory, Department of Zoology, University of Toronto, Toronto, Ont.

[2] *Graduate Student, Department of Zoology, University of Toronto.*

much oxygen as the blood of the catfish and four times as much as the carp for equal volumes of blood circulated. Such differences in the blood should confer on each species a distinction in the transport of oxygen according to its carbon dioxide sensitivity, and it was thought that these differences might be reflected in differences in ability of the fish to utilize oxygen. However, measure of the maximum rates of oxygen transport (2) showed that the yellow perch, which was sensitive to carbon dioxide, consumed oxygen at a lower rate than the catfish, which had blood tolerant to carbon dioxide. Hence it seemed evident that factors other than the properties of the blood were involved in transport of oxygen.

An investigation of the cardiac output and beat rate of the catfish, carp, bowfin, and common sucker was carried out in order to determine the oxygen transport capacity from the mechanical standpoint. Since, as shown by Black (1), the chemical aspects of the bloods of these four species were such as to confer large advantages to certain of them in the transport of oxygen, the same species were studied here to determine whether or not certain mechanical aspects of the circulation were more advantageous to some species than to others.

Method

The fish used were bought from live-fish dealers in Toronto during the months from December, 1939, to April, 1940. The catfish, bowfin, and carp were caught largely in Lake Erie, whereas most of the suckers were obtained from Lake Simcoe. Before being used, the fish were kept in tanks for various periods of time, from a few hours to a week, at approximately 10° C. The experiments were carried out on 48 catfish, 43 bowfins, 40 carp, and 37 suckers.

In preparation for an experiment, the fish was stunned by blows on the head and the beating heart was exposed by cutting through the ventral body wall and pericardium. This and subsequent operations were carried out at room temperature. Ligatures were then placed loosely around the bulbus arteriosus and atrioventricular passage flush with the ventricle. These were tightened to isolate the quantity of blood contained in the ventricle from the rest of the circulatory system. After ligation, the ventricle was removed and weighed with the contained blood. The ligatures and adhering portions of the atrium and bulbus were carefully trimmed off and their weight subtracted from the first weight. The ventricle was then opened, washed thoroughly, hardened in 10% formalin for several hours, squeezed dry, and weighed again. This procedure was adopted since it was found possible to remove from 30 to 60% more fluid from the spongy inner surface of the ventricle when so treated than could be squeezed from the fresh material without damage to it. It was found that wet ventricles weighed fresh and also after being hardened in formalin showed no significant difference in weight, so that hardening caused no change in weight of the muscle itself.

Four different methods were used for ligating the ventricle during diastole. The first consisted in tightening the two ligatures simultaneously in the

interval between the contraction of the atrium and the ventricle. In many fish a modification of the above technique was used; in this the atrial ligature was tightened following the next succeeding contraction. A third variation was sometimes used for suckers; in these the heart-beat rate was often high and it was necessary to insert a cannula into the bulbus to relieve the pressure before the ligatures were tied; this will be referred to later (page 80). Finally, the hearts were sometimes poisoned with potassium nitrate to cause them to stop beating in diastole (5). Except in the bowfin, all these methods of preparation gave similar results. The quantities of blood in the ventricles were greater when the heart of the bowfin was poisoned with potassium than when it was not; hence these values have not been considered in the average although they are plotted on the graph (Fig. 1).

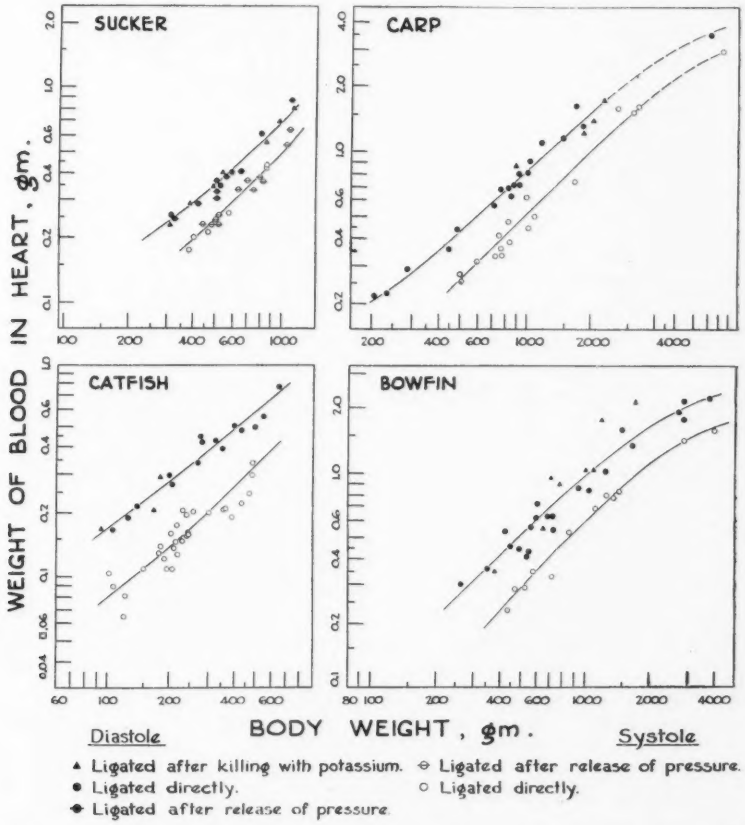


FIG. 1. *Curves showing the weight of the blood in the ventricle in diastole and systole of the bowfin, sucker, carp, and catfish as a function of body weight, double logarithmic grid.*

In measuring the blood content during systole, the ligatures were for the most part tied simultaneously, immediately following ventricular contraction. Occasionally the atrial ligature was tied one, two, or three beats before the bulbus ligature. In suckers with high beat rate the ventricles were ligated after release of pressure by the insertion of a cannula (see below). All these methods gave similar results.

There is a difference in the accessibility of the hearts of the four species. The hearts of the bowfin and the sucker are readily exposed, whereas the hearts of the catfish and carp are exposed with difficulty because of the presence of the bones of the pectoral girdle. In the latter two species more shock resulted than in the former two. The shock of exposing the heart was reflected in some fish by an acceleration in beat rate, in others by a retardation. In extreme cases, the exposure resulted in the cessation of the atrial beat for short periods; this was especially marked in the catfish.

The dynamics of the heart-beat are affected by various factors. It is well known that variations occur in the stroke output of the hearts of higher vertebrates with changes in beat rate. Such changes in rate commonly occurred in the fish hearts when they were being exposed, and when the ligatures were being placed in position for tying. In the sucker, the effect of stunning and of struggling on the part of the fish usually caused a marked acceleration of the beat rate from an average of 20 up to 35 or 40 beats per minute. In such fish, the bulbus and ventricle often became greatly distended with blood, and it was found that when ligatures were tied directly there was often more blood in the ventricle at systole than there was normally at diastole (i.e. when the rate was low). However, when a cannula was inserted into the bulbus to allow the blood excess to escape and relieve the pressure before the ligatures were tied, the results were similar to those obtained by the other methods. The rate of the heart-beat of a representative sample of the fish under the conditions of the study was determined and is recorded in Table I.

Results

In Fig. 1, the weight of the blood contained in the ventricle at diastole and at systole is plotted against body weight on a double logarithmic grid. Each point represents one fish. The difference between corresponding points on the diastolic and systolic curves for one species represents the weight of blood passed per beat, the stroke weight or *stroke output* under the experimental conditions. These differences are plotted against the body weight in Fig. 2. It may be seen that within each species, the stroke output increases as the body weight increases. The size range differed in the specimens used but the four species may be compared over the range from 300 to 600 gm. At 500 gm., the catfish has the largest cardiac output per beat, i.e., 0.26 gm. of blood (Table I); at this weight the bowfin has an output of 0.22 gm., the carp 0.18 gm., and the sucker 0.11 gm. Although the stroke output increases as the body weight increases, the output per unit weight decreases as the body weight increases (Fig. 3).

TABLE I

HEART-BEAT RATE, STROKE OUTPUT, MEAN MINUTE OUTPUT, AND VENTRICLE WEIGHT IN THE CATFISH, CARP, BOWFIN, AND SUCKER, AND COMPARISON WITH PRESSURES OF OXYGEN REQUIRED FOR HALF SATURATION AND WITH THE CARBON DIOXIDE EFFECT UPON BLOOD

	Catfish	Carp	Bowfin	Sucker
CO_2 effect, rise* in pO_2, in mm. Hg, due to 1 mm. CO_2	0.25	1.0	1.4	4.0
pO_2, in mm. Hg, at 50% HbO_2* with 0-2 mm. pCO_2	1.4	5.0	4.0	12.0
Stroke output, gm., at 500 gm. body wt.	0.26	0.18	0.22	0.11
Heart rate**, beats per minute	21.6 ± 4.3 (17 fish)	21.1 ± 3.1 (10 fish)	13.9 ± 2.5 (20 fish)	20.4 ± 1.3 (8 fish)
Mean minute output, gm., under experimental conditions	5.50	3.80	3.06	2.24
Ventricle weight, gm., at 500 gm. body weight	0.185	0.170	0.180	0.175

* *From Black (1).*

** *In the bowfin, catfish, and the carp the values for beat rate are recorded from fish selected at random. The beat rate for suckers is the mean after excluding the fish in which the bulbus and atrium were distended with blood.*

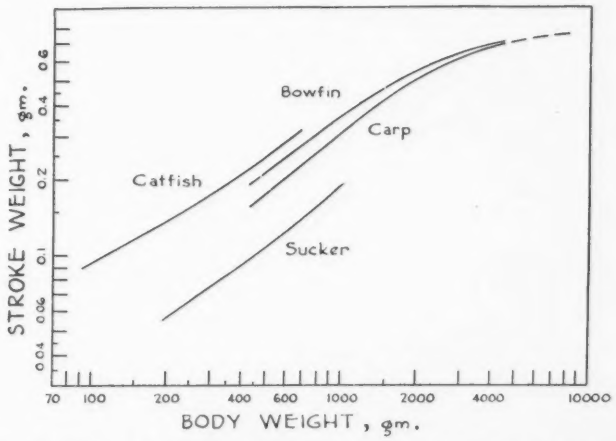


FIG. 2. *The relation between stroke output and body weight of the bowfin, sucker, carp, and catfish, double logarithmic grid. The stroke output was obtained by subtracting corresponding points on the systolic from the diastolic curves for each species in Fig. 1.*

The mean beat rate measured after exposure of the heart was found not to vary greatly from species to species (Table I). The values of the minute output shown in this table, obtained by multiplying the beat rate by the corresponding cardiac output per beat, are greatest for the catfish and are followed in turn by those of the carp, the bowfin, and the common sucker.

The cardiac output does not appear to be correlated with the mass of the ventricular muscle. At 500 gm. body weight there is little difference in the

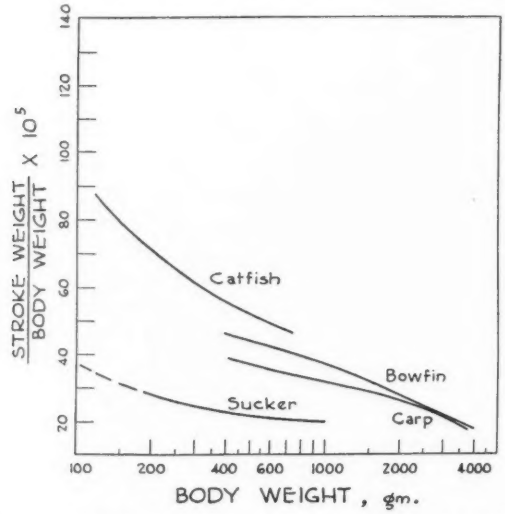


FIG. 3. *The relation between stroke output per unit weight and body weight of the bowfin, sucker, carp, and catfish, semilogarithmic grid.*

weight of the ventricle in each species although there are large differences in output (Table I). On the other hand, the differences in output appear to be related to the magnitude of the expansion and contraction of the ventricle through the cardiac cycle. Transverse sections indicate that during diastole the ventricle of the catfish has a larger cavity than that of the sucker (Figs. 4 and 5).

Discussion

Although the determinations were made under abnormal conditions on fish out of water, it is believed that the results obtained give a good approximation of the magnitude of the cardiac output in the four species. The hearts were usually beating regularly and circulating blood, and the regularity of the relation of blood weight to body weight at systole and diastole suggests the significance of the measurements as an indication of what may happen under normal conditions. The determination of the output under natural conditions was not the purpose of this investigation, but rather the determination of the specific differences as they existed under the experimental conditions.

The method described above has been applied to similar species in other localities and very similar results have been obtained. For example, both the catfish examined at Toronto and catfish investigated at Welaka, Florida (3), had a mean stroke output of 0.27 gm. of blood at 500 gm. body weight. Suckers taken from the Delaware River at Philadelphia in December, 1940, had a stroke output of 0.13 gm. of blood at this weight, the suckers examined at Toronto had an output of 0.11 gm.

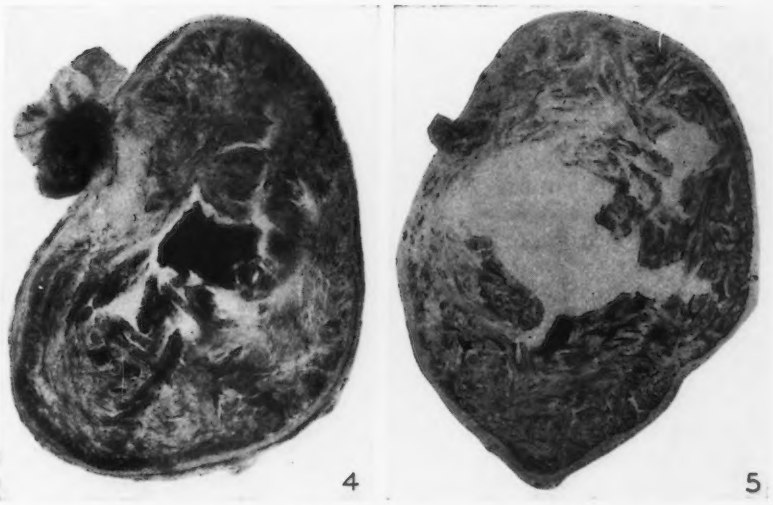


FIG. 4. *Celloidin section through maximum diameter of the sucker ventricle in diastole, to illustrate relation between muscle volume and cavity volume. ×7. Weight of fish, 465 gm. Beat rate, 21 per minute. The dark mass in the cavity is blood. A portion of the atrioventricular tissue can be seen attached to the ventricle.*

FIG. 5. *Celloidin section through the maximum diameter of the catfish ventricle in diastole, to illustrate the relation between muscle and cavity volume. ×7. Weight of fish, 471 gm. Beat rate, 21 per minute. The blood is not present in the main cavity but can be seen as dark areas scattered throughout the section.*

It follows that for equal beat rates of the heart, the amount of blood circulated in a given time varies in different fish. This would tend to cause differences in the oxygen transport in the various species. The minute output of the four species studied is such as to confer on the catfish an advantage over the remaining species in that it circulates blood about one and one-half times as fast as the carp, almost twice as fast as the bowfin, and about two and one-half times as fast as the sucker, under the experimental conditions. A similar relation may exist under natural conditions.

The carbon dioxide sensitivity of the bloods of the four species studied by Black was such as to confer on the sucker a large advantage over the catfish in the unloading of oxygen to the tissues, and hence by virtue of this property of the blood, this fish should be able to transport more oxygen than the catfish for equal volumes of blood circulated. When the minute output and the carbon dioxide sensitivity of the blood are compared (Table I), they are seen to vary inversely. In the fish studied in Florida, a similar situation was found. In each species the respiratory tolerance to carbon dioxide was studied instead of the carbon dioxide sensitivity of the blood. Those fish that were tolerant to carbon dioxide (four species) had a larger cardiac output than others that were sensitive to carbon dioxide (two species). The blood

pressure (bulbus arteriosus) was also found to vary in the Florida species, those with a large stroke output possessing a lower blood pressure than others with a small output.

It thus appears that differences in both the chemical and mechanical transport of oxygen have to be considered in order to explain similarities or dissimilarities in the oxygen consumption by different species. That these two factors may compensate for each other to some extent has been indicated above. Since species with blood of high sensitivity to carbon dioxide do not necessarily consume oxygen at a faster rate than those with blood of low sensitivity to carbon dioxide (2), the circulation may be paramount in compensating for differences in transport of oxygen by virtue of the carbon dioxide effect. However, other differences in the chemical properties of the bloods of these species may compensate for differences in carbon dioxide effect. For example, the inverse relation between the carbon dioxide effect on blood and the affinity of the same blood for oxygen was demonstrated in these species by Black (1) and is shown in Table I.

The relation of the bloods of the four species to the environments in which they lived was pointed out by Black. Blood with high sensitivity to carbon dioxide and low affinity for oxygen was associated with cold water habit (common sucker), and blood with low sensitivity and high affinity for oxygen was associated with warm water habit (catfish). Thus the former species is found in the deep water in Algonquin Park lakes during summer while the latter is found in shallow surface water. Similarly, small cardiac output may be associated with cold water habit and large output with warm water habit in these lakes.

Acknowledgments

Sincere thanks are due Dr. F. E. J. Fry who suggested the problem, and who was most liberal with his time and guidance during the course of the work.

The writer would also like to express thanks to Professors E. Horne Craigie and W. J. K. Harkness for advice, and to Dr. K. C. Fisher for use of equipment in the laboratory of experimental biology. The writer is grateful to Dr. Laurence Irving of Swarthmore College, Pa., for criticism of the manuscript.

References

1. Black, E. C. The transport of oxygen by the blood of freshwater fish. Biol. Bull. 79 : 215-229. 1940.
2. Black, E. C., Fry, F. E. J., and Scott, W. J. Maximum rates of oxygen transport for certain freshwater fish. Anat. Record, 75 (Suppl.) : 80. 1939.
3. Hart, J. S. The circulation and respiratory tolerance of some Florida freshwater fish. Proc. Florida Acad. Sci. (In press).
4. Krogh, A. and Leitch, I. The respiratory function of the blood in fishes. J. Physiol. 52 : 288-300. 1919.
5. Mathison, G. C. The effects of potassium salts upon the circulation and their action on plain muscle. J. Physiol. 42 : 471-494. 1911.
6. Root, R. W. The respiratory function of the blood of marine fishes. Biol. Bull. 61 : 427-456. 1931.
7. Willmer, E. N. Some observations on the respiration of certain tropical freshwater fishes. J. Exptl. Biol. 11 : 283-306. 1934.

CANADIAN WILTSHIRE BACON

XXIII. THE EFFECT OF CONCENTRATION OF CURING SALTS ON COLOUR AND COLOUR STABILITY[1]

By A. H. Woodcock[2] and W. Harold White[3]

Abstract

Small cuts of pork back were cured in pickles containing six concentrations each of chloride, nitrate, and nitrite varying from zero to above the concentrations normally used commercially. Quantitative colour measurements were made by an improved three-colour method on the internal surfaces of the lean meat. Salt contents were determined for each sample.

Increase of chloride concentration retarded methaemoglobin formation as indicated by a relative increase in the green and corresponding decrease in the red colour components. Variations in the nitrate content of the meat appeared to have no significant effect on the colour. The presence of nitrite in the meat caused the appearance of an absorption band in the region of 490 mμ, and a retardation of methaemoglobin formation as indicated by the red and green components of the initial colour and also by colour stability. The absorption band at 490 mμ is considered to be due to nitrosohaemoglobin. The presence of approximately 50 p.p.m. of nitrite in the meat appears to have been sufficient for complete reaction.

Introduction

The characteristic colour of bacon is attributed primarily to nitroso compounds formed by the reaction of nitrite with blood and muscle pigments of the haemoglobin type (1, 3, 5, 8, 10). Chloride and nitrate, the other two salts normally used in curing Wiltshire bacon, are reported to have some effect also on the resulting colour (2, 4, 7, 9).

Relations between the chloride, nitrate, and nitrite contents of commercially cured bacon and quantitative measurements of the colour by means of a photoelectric comparator provided with three filters have been reported previously (13, 14). However, the small differences observed in the contents of the curing salts made definite estimation of their effect on colour difficult (6). Moreover, a recent study has shown that a more suitable evaluation of the colour of bacon may be obtained by the use of a photoelectric colour comparator employing a new principle in the separation of the three colour bands (15). The present paper describes an investigation in which quantitative measurements by the improved method were made of the colour quality and stability of laboratory-cured bacon containing widely varying amounts of chloride, nitrate, and nitrite.

[1] *Manuscript received January 21, 1943.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 87 of the Canadian Committee on Food Preservation, and as N.R.C. No. 1107.

[2] *Biophysicist, Food Preservation Investigations.*

[3] *Biochemist, Food Preservation Investigations.*

Material and Methods

The material consisted of one full-length, rib-in, export pork back taken from each of 18 hogs specially selected as to uniformity of size and quality. Three pieces approximately 5 in. in length were cut from the front end of each back. One sample was allotted at random to each of the various curing treatments.

The investigation was divided into two parts in order to obtain a sufficient range of conditions without the use of an impractically large number of samples. The first part contained all possible combinations of 0, 15, and 30% sodium chloride, 0, 1, and 5% sodium nitrate, and 0, 0.1, and 0.5% sodium nitrite; and the second part, all possible combinations of 5, 10, and 20% sodium chloride, 0.5, 3.0, and 10% sodium nitrate, and 0.05, 0.2, and 1.0% sodium nitrite. Each sample was cured for 70 hr. at 4.4° C. (40° F.), after which it was wiped, drained for 24 hr., wrapped in waxed paper, and matured for nine days at 7.1° C. (45° F.).

The chloride, nitrate, and nitrite contents of each sample were determined by methods previously described (12). The colour of the freshly cut surface of the lean meat was measured with a photoelectric comparator (15). Brightness was determined as the total amount of light scattered by the sample as compared with a standard white surface. Colour quality was estimated as the percentage distribution of the total light scattered by the sample in the blue, green, and red bands, namely, 464 to 525 mμ, 525 to 600 mμ, and above 600 mμ, respectively.

In order to obtain an estimate of colour stability, the sample, after the initial measurement, was exposed to the atmosphere for 70 hr. at 7.1° C. and 95% relative humidity, and its colour remeasured.

Results and Discussion

Initial Colour

Mean values for colour and for the concentrations of sodium chloride, nitrate, and nitrite in the bacon at each concentration of the curing pickle are shown in Table I. An increase in the concentration of sodium chloride was associated with an increase in the red scatter, and a decrease in both the green scatter and total brightness. The blue intensity was relatively unaffected by change in the chloride concentration.

Statistical analyses were used to assess the significance of the observed changes. By means of partial correlation coefficients it was possible to ascertain the independent effect of each salt on the colour of pork. It will be seen from Table II that the changes with chloride concentration in the red and green intensities and in total brightness were statistically significant.

The increase in red and corresponding decrease in green intensity are presumably due to a retardation of methaemoglobin formation as the concentration of chloride is increased. It has been reported that chloride, when present in large amounts, markedly accelerates the formation of methaemo-

TABLE I

MEAN VALUES FOR THE COLOUR AND CONCENTRATION OF CURING SALTS IN BACON

Curing salt	Concentration of curing salts		Scatter, %			Brightness, %
	Pickle	Bacon	Blue	Green	Red	
Sodium chloride	0 %	0 %	34.8	33.3	31.9	18.2
	5	1.29	35.2	33.4	31.4	16.0
	10	2.12	34.5	33.6	31.9	14.6
	15	2.98	34.5	33.0	32.5	15.7
	20	3.80	34.6	32.9	32.5	13.5
	30	5.14	34.4	32.5	33.1	14.6
Sodium nitrate	0 %	0 %	35.0	32.4	32.6	16.3
	0.5	0.04	34.5	33.2	32.3	14.6
	1.0	0.06	34.3	33.2	32.5	16.7
	2.0	0.29	35.0	33.3	31.6	15.0
	5.0	0.52	34.5	33.2	32.3	15.6
	10.0	1.45	34.9	33.1	32.0	14.5
Sodium nitrite	0 p.p.m.	4 p.p.m.	36.0	32.4	31.6	16.6
	500	59	34.8	33.3	31.9	15.7
	1000	96	33.7	33.2	33.1	15.4
	2000	202	34.8	32.9	32.3	14.2
	5000	407	34.1	33.2	32.7	16.6
	10000	1046	34.9	33.4	31.7	14.2

TABLE II

PARTIAL CORRELATION COEFFICIENTS BETWEEN COLOUR AND THE CONCENTRATION OF CURING SALTS IN BACON

Quantities correlated	Scatter			Brightness
	Blue	Green	Red	
Chloride independent of nitrate and log nitrite	0.04	−0.34*	0.47**	−0.59**
Nitrate independent of chloride and log nitrite	0.18	0.02	−0.14	−0.21
Log nitrite independent of chloride and nitrate	−0.48**	0.19	0.31*	−0.34*

* *Indicates 5% level of statistical significance.*
** *Indicates 1% level of statistical significance.*

globin (4). The results of the present investigation suggest that this conclusion is not valid for such concentrations of sodium chloride as are normally present in Wiltshire bacon. The changes in total brightness show that the meat becomes darker in colour with increase in chloride concentration.

Nitrate had little effect on the colour of bacon (Tables I and II). While the total brightness in general decreased with increase in the concentration of nitrate, the differences were not statistically significant.

An increase in the concentration of nitrite in the meat caused a reduction in the blue intensity and in brightness, and an increase in the red intensity (Table I). The amount of light scattered in the green was relatively unchanged. Partial correlation coefficients showed that the observed changes in the blue and red scatter, and in brightness were statistically significant (Table II). Since the relation between nitrite concentration and colour scatter is obviously not linear (Table I), logarithms of the nitrite content were employed in these computations as a closer approximation to the actual function.

The major portion of the observed changes occurred in the blue region and for concentrations of nitrite in the meat of up to approximately 50 p.p.m. If the colour changes are attributed to a chemical reaction of nitrite with the meat pigment, then the indications are that the reaction is complete when an excess of approximately 50 p.p.m. of nitrite is present. The compound formed has an absorption band in the blue region (464 to 525 mμ), in agreement with previous investigations (11, 15). In addition, the compound is more resistant to methaemoglobin formation than the normal meat pigments since the red scatter is increased by the addition of nitrite.

There is some indication that nitrite, like chloride, reduced the brightness of the meat. Since the values are variable, however, it is unknown whether this reduction is due to the chemical reaction of the nitrite with the meat pigments, causing a reduction of the blue scatter, or to a more general reduction in brightness comparable with the effect of chloride.

Colour Stability

The changes on exposure to air in colour quality and brightness were generally small and irregular. An analysis of variance of these changes according to pickle composition revealed that nitrite was the most important of the three curing salts in contributing to colour stability (Table III). It appears that the change in colour is due to methaemoglobin formation, and that the reaction product of nitrite is more resistant to such a change than the normal meat pigments. The observed decrease in brightness on exposure bore no statistically significant relation to the various cures.

TABLE III

MEAN VALUES FOR THE EFFECT OF NITRITE ON THE COLOUR STABILITY OF BACON

Nitrite in curing pickle	Scatter, %			Brightness, %
	Blue	Green	Red	
Present	0.0	+2.5	−2.5	−0.28
Not present	−0.7	+4.3	−3.6	−0.26

Acknowledgments

The authors wish to thank Dr. J. W. Hopkins, Statistician, National Research Laboratories, for his advice on the statistical computations, and Messrs. H. Tessier, M. L. McMahon, and D. A. Wright, Laboratory Assistants, for their technical assistance.

References

1. ANON. Food Manuf. 12 : 230-231. 1937.
2. BROOKS, J. Proc. Roy. Soc. (London) B, 109 : 35-50. 1931.
3. BROOKS, J. Proc. Roy. Soc. (London) B, 123 : 368-382. 1937.
4. BROOKS, J. Food Research, 3 : 75-78. 1938.
5. BROOKS, J., HAINES, R. B., MORAN, T., and PACE, J. Dept. Sci. Ind. Research (Brit.), Spec. Rept. Food Investigation Board, No. 49. 1940.
6. COOK, W. H. and WHITE, W. H. Can. J. Research, D, 18 : 135-148. 1940.
7. HOAGLAND, R. U.S. Dept. Agr., Ann. Rept. Bur. Anim. Ind., 1908, pp. 301-314. 1910.
8. JONES, O. Food, 6 : 238-240. 1937.
9. SIDWELL, A. E., JR., MUNCH, R. H., BARRON, E. S. G., and HOGNESS, T. R. J. Biol. Chem. 123 : 335-350. 1938.
10. URBAIN, W. M. and JENSEN, L. B. Abstract. Food Manuf. 12 : 377. 1937.
11. URBAIN, W. M. and JENSEN, L. B. Food Research, 5 : 593-606. 1940.
12. WHITE, W. H. Can. J. Research, D, 17 : 125-136. 1939.
13. WINKLER, C. A., HOPKINS, J. W., and ROOKE, E. A. Can. J. Research, D, 18 : 225-232. 1940.
14. WINKLER, C. A., HOPKINS, J. W., and THISTLE, M. W. Can. J. Research, D, 18 : 217-224. 1940.
15. WOODCOCK, A. H. Can. J. Research, D, 21 : 90-97. 1943.

COLOUR OF MEAT

IV. MEASUREMENT OF THE COLOUR OF BACON[1]

By A. H. Woodcock[2]

Abstract

Spectrophotometric measurements were used to study the reflection spectrum of pork and bacon in detail. A new method of colour measurement using nine filters was developed and tested experimentally on 100 samples of bacon treated in various ways.

It was found that three distinct changes could occur in the colour quality of bacon which were related to known physical or chemical changes. A loss of scatter in the blue region was associated with the reaction between nitrite and the haemoglobin pigments. An increase of scatter in the green with a corresponding decrease of scatter in the red was interpreted as loss of haemoglobin and a corresponding increase of methaemoglobin pigment.

These changes normally occurring in the colour of bacon can be assessed with three filters which absorb light below wave lengths of 460, 520, and 595 mμ, thus simplifying the method without loss of efficiency.

The estimation of the total scatter of the bacon is made by an independent measurement.

Introduction

A comparator for estimating objectively the colour of meat or other similar materials has been described in earlier papers of this series (6, 8). The instrument was based on the standard tricolour method of defining colour in the blue, green, and red spectral regions. These were isolated by filters. The values for light scattered in each spectral region were expressed as a percentage of the scatter from a standard white surface in the same region. The sum of the scatters by each component yielded a relative estimate of brightness.

Theoretically this comparator was capable of estimating two attributes of colour, chroma or colour quality and brightness. Practical experience (3, 4, 7, 9, 10, 11, 12, 13) demonstrated that the instrument was entirely satisfactory for studying changes that affected brightness primarily (3, 4, 7). It was less satisfactory for estimating colour quality since the scatter values for meat in the three broad regions of the visible spectrum studied were generally correlated (10). In addition it was found difficult to demonstrate that independent fluctuations significantly exceeded the experimental and sampling errors.

The present investigation was undertaken to determine the wave bands required to specify the colour of bacon, their width, number, and position in the visible spectrum, and the method most suitable for measuring these bands and interpreting the results.

[1] *Manuscript received January 21, 1943.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 88 of the Canadian Committee on Food Preservation and as N.R.C. No. 1108.

[2] *Biophysicist, Food Investigations.*

Spectrophotometric Examination

The first phase of the investigation consisted of a detailed spectrophotometric examination of the light scattered from the surface of fresh pork and of smoked and unsmoked bacon, to determine the width and position of those regions of the visible spectrum in which colour changes occur. The spectrophotometer, equipped with a Hilger constant deviation spectrometer as monochromator, was used with a resolving power of 6 mμ in the region of 589 mμ. Observations were made in the visible spectrum at 5 mμ intervals from 425 mμ in the violet to 680 mμ in the red. All measurements were expressed as a percentage of the light scattered in the same region by a standard white surface of magnesium carbonate.

Typical curves for fresh pork and for unsmoked and smoked bacon, shown in Fig. 1, serve to demonstrate the principal features of the colour. The

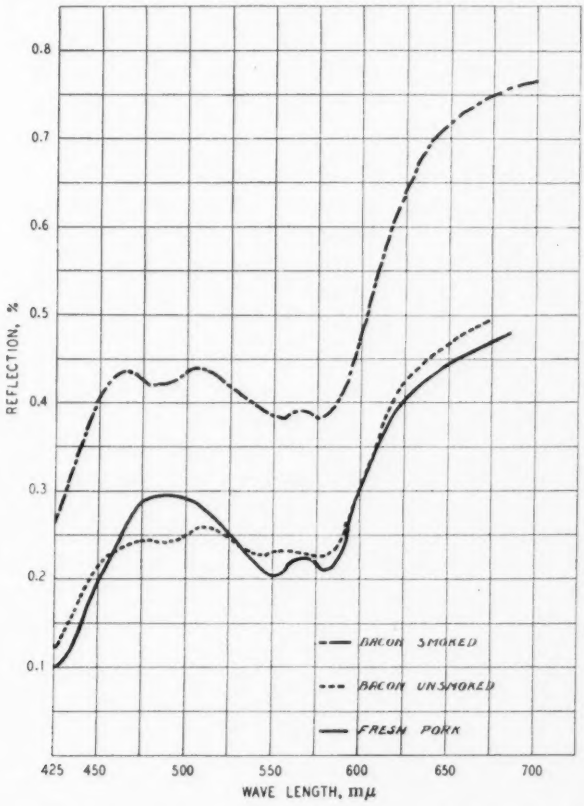


FIG. 1. *Typical spectrophotometer curves for fresh pork and unsmoked and smoked bacon.*

minima at 545 mμ and at 575 mμ are common to all three curves and in addition were found to be present in diluted pigs' blood. They correspond to those reported by Sidwell and others (5) for oxyhaemoglobin, and also to those reported by Brooks (1) for nitrosohaemoglobin. Consequently they were interpreted as absorption bands characteristic of both oxy- and nitrosohaemoglobin. Bacon in contrast to pork has an absorption band centred near 490 mμ which is considered to be characteristic of nitrosohaemoglobin (14).

The effect of smoking was to increase the amount of light scattered without changing the contour of the curve to any great extent. Hence curing affected the colour quality of the meat, while smoking, on the other hand, changed the brightness.

Spectrophotometric measurements by this method were not suited for extensive work on inherently variable biological materials where a large number of observations are necessary to reach valid conclusions. The resolving power of the spectrophotometer was unnecessarily high since observed changes in the scatter were due to absorption bands of the order of 25 mμ in width. Further it is doubtful if, under normal conditions, the eye could detect a differential change in the colour of meat between two wave lengths of less than this amount.

Examination by Filter Methods

Three Filter Method

The spectrophotometric transmission curves of the blue, green, and red filters used in the three colour comparator are shown in Fig. 2. From these curves it is evident that the comparator was relatively insensitive to changes in scatter in the regions near 485 mμ and 575 mμ, the bands indicated by spectrophotometric measurements to be of greatest importance.

It is a well known property of filters that the lower wave length limit is much sharper than the upper. This is illustrated by the curves in Fig. 2 where the green filter cuts off quite sharply on the short wave length side at 500 mμ but tapers gradually from 540 to 580 mμ on the long wave length side. Such filters are unsuitable for isolating bands of the order of 50 mμ in the visible spectrum. More selective filter combinations are available but are so dense that they transmit an insufficient amount of light to operate the photocell.

Nine Filter Method

From the above considerations the requirements of a comparator yielding more information on the colour could be estimated. Such requirements were that the width of any colour band be not more than 50 mμ, all portions of the spectrum be emphasized as equally as possible, the method be reasonably rapid so that large numbers of samples could be studied, and if possible that the measurements be made in such a manner that the results could be easily interpreted in terms of colour quality and brightness.

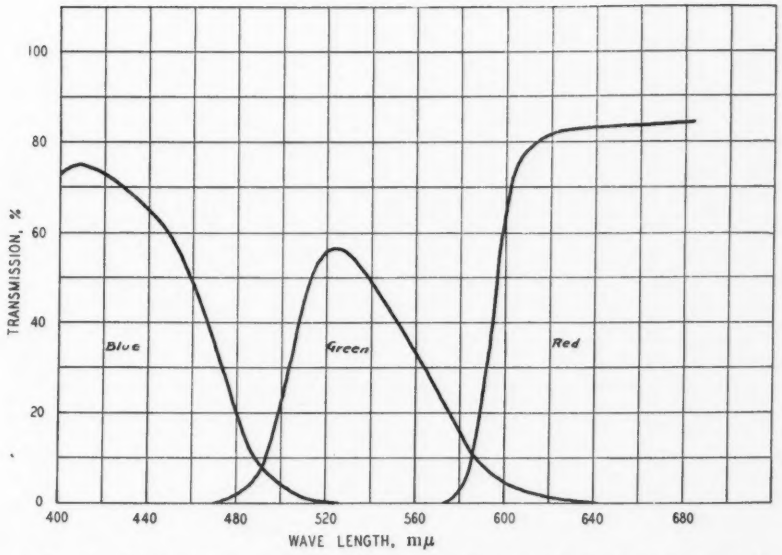


FIG. 2. *Spectrophotometric transmission curves of filters used in the three colour method.*

A new method for using light filters was chosen to fulfil these requirements. Nine filters of the type that transmits only light above certain characteristic wave lengths were arbitrarily selected to cover the entire visible spectrum at approximately equal intervals. From the transmission curves of these filters, as shown in Fig. 3, it will be seen that all had sharp cut-offs distributed over the entire visible spectrum. To measure brightness the scatter from the sample was compared with and expressed as a percentage of the scatter from a standard white surface using the filter transmitting all visible light.

Colour quality was measured by successively cutting off portions of the light scattered by the sample. By subtracting adjacent observations eight differences were obtained which, together with the transmission through the last dark red filter, made up 100% of the scattered light. The bands so formed are represented in Fig. 4. The scatter in each band was thus expressed as the percent of total scatter.

This satisfied the requirements mentioned above. The bands were all less than 45 mμ in width and were such that equal sensitivity was obtained over the whole visible spectrum. The colour quality was measured independently of the brightness.

For these experimental tests a temporary apparatus was used in which one of the three colour comparators was modified to permit the use of nine filters and a second comparator employed as a compensating source to eliminate the fluctuations of light intensity caused by variations in line voltage. Such compensation was found necessary since measurement of differences in

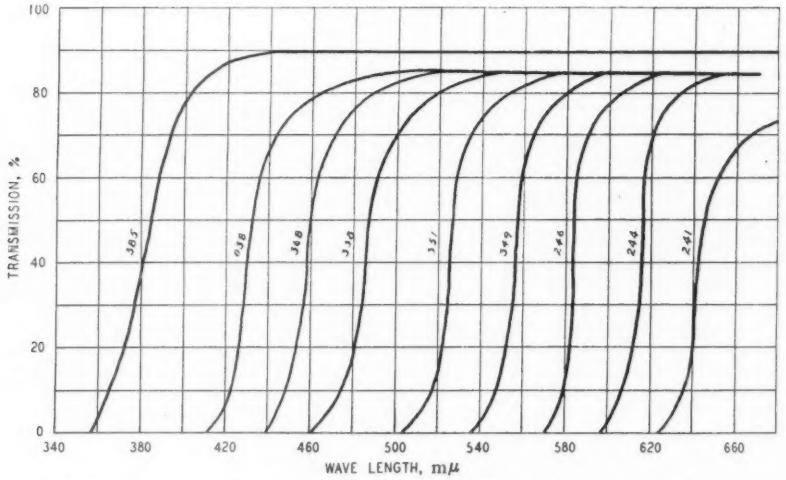


FIG. 3. *Spectrophotometric transmission curves of filters used in the nine colour method.*

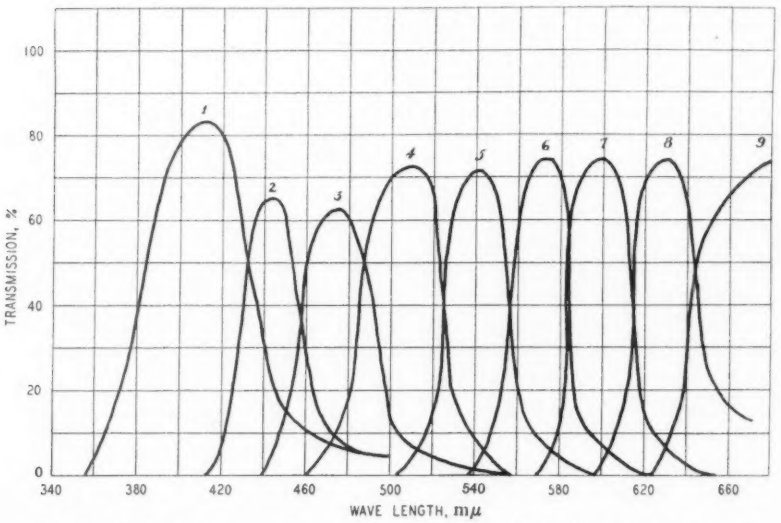


FIG. 4. *Spectrophotometric curves of the resultant colour bands obtained when using the nine colour method.*

scatter amplified the instrumental errors. The compensating circuit consisted of a fixed resistor placed in parallel with the photocell of the nine colour comparator and a calibrated variable resistor likewise in parallel with the compensating photocell. The voltages developed across these resistors were balanced using a galvanometer as a null instrument.

This type of apparatus, while it proved satisfactory for test purposes, could be greatly improved. The use of vacuum type photocells in a modern balanced electronic circuit, and a calibrated diaphragm to equalize the intensity of the light on the photocells would offer considerable improvement.

Comparison of Three and Nine Filter Methods

The relative ability of the three and nine colour methods to distinguish between colour quality and brightness was studied on two groups of samples. The first group, selected to illustrate changes due to methaemoglobin formation, consisted of 50 samples of smoked bacon selected at random from other studies in which storage conditions only were varied. The second group, considered to illustrate both methaemoglobin and nitrosohaemoglobin formation, consisted of 50 samples of unsmoked bacon, 27 of which were known to have variations in colour due to differences in curing pickles, the other 23 being chosen at random from a large number varying in either cure or storage history.

The experimental error for each band was determined by making duplicate measurements. The samples were removed after each measurement so that this error would include variations in the instrument and in the orientation of the bacon in the sample holder.

Results

Correlation coefficients were computed between the light scattered in each colour band by the three filter method, and the brightness measured by the new method (Table I). It may be seen that the coefficients are very high, indicating that approximately 90% of the variance encountered with the three colour instrument is due to brightness, leaving the remaining 10% to account for changes in colour quality, independent variations, and error. This supports the statement that the three filter method indicated mainly changes in brightness.

TABLE I

CORRELATION OF BRIGHTNESS AS MEASURED BY THE NINE COLOUR METHOD WITH COLOUR AS DETERMINED BY THE THREE COLOUR METHOD

Quantities correlated	Smoked bacon		Unsmoked bacon	
	D.f.	r	D.f.	r
Brightness × blue	48	+ 0.96	48	+ 0.94
Brightness × green	48	+ 0.96	48	+ 0.96
Brightness × red	48	+ 0.93	48	+ 0.93

Data obtained by the nine filter method were reduced by computing the standard deviations and standard errors of each band and the correlation coefficients between adjacent bands as shown in Table II. It may be seen that in the majority of the bands the standard deviation or variability of the

material measured exceeded the instrumental errors, indicating that the method has adequate precision.

TABLE II

COLOUR QUALITY OF BACON—INSTRUMENTAL ERROR, STANDARD DEVIATION, AND CORRELATION BETWEEN ADJACENT BANDS AS MEASURED BY THE NINE COLOUR METHOD

Band	Mean square error (19 deg. freedom)	Smoked bacon				Unsmoked bacon			
		Standard deviation (49 deg. freedom)	Bands correlated	D.f.	r	Standard deviation (49 deg. freedom)	Bands correlated	D.f.	r
1	7.8	26.5**	1 and 2	48	0.001	15.9**	1 and 2	48	−0.03
2	5.3	31.9**	2 and 3	48	0.01	29.7**	2 and 3	48	−0.05
3	5.4	8.6*	3 and 4	48	0.01	17.2**	3 and 4	48	0.36*
4	5.0	15.7**	4 and 5	48	0.69**	39.6**	4 and 5	48	0.18
5	1.5	15.6**	5 and 6	48	0.87**	30.2**	5 and 6	48	0.77**
6	2.4	74.0**	6 and 7	48	−0.63**	101.3**	6 and 7	48	−0.74**
7	1.5	16.6**	7 and 8	48	0.84**	33.2**	7 and 8	48	0.81**
8	3.1	31.6**	8 and 9	48	0.93**	41.9**	8 and 9	48	0.70**
9	1.8	33.2**				56.2			

* *Exceeds 5% level of significance.*
** *Exceeds 1% level of significance.*

In assessing the significance of the data obtained for the light scattered in the various bands it should be noted that a high correlation coefficient between adjacent colour bands indicates that the same change was observed in each. Correlations between Bands 1 and 2, 2 and 3, 3 and 4 for smoked bacon and between Bands 1 and 2, and 2 and 3 for unsmoked bacon were small, indicating that the variations, though exceeding the standard error, were independent. Since it seems unlikely that the filters arbitrarily selected should be such that they coincided with regular independent colour changes, it appears that inherent variations between samples were being detected.

High positive values of the correlation coefficients were obtained between Bands 4 and 5, 5 and 6, and between Bands 7 and 8, and 8 and 9; the negative coefficient between Bands 6 and 7 indicated that two related types of change occurred. Examination of the standard deviations showed that Bands 6 and 9 were most affected by the changes. The colour Bands 4, 5, and 6 can be identified by comparison with the spectrophotometric curves as being due to haemoglobin while Bands 7, 8, and 9 cover the region of methaemoglobin absorption (2). Thus both changes were caused by the oxidation of haemoglobin to methaemoglobin.

Examination of the results of colour measurements on unsmoked bacon indicated another type of change as well as that associated with methaemoglobin formation. This was indicated by a higher correlation between Bands 3 and 4 than between adjacent bands. Spectrophotometric studies, as mentioned previously, indicated that an absorption band occurred in this region and that it is formed as a result of curing.

Discussion

The results obtained above indicate that variations in the colour quality of bacon resulting from variations in curing or storage procedure can be assessed with three filters, thereby simplifying the method. The effect of other treatments on colour of bacon would have to be examined fully if it were suspected that they caused other types of colour change.

The nature of these changes led to the selection of the following three filters for the specification of colour. Since Bands 1 and 2 did not appear to be related to any specific change and are also in the extreme violet region of the spectrum where the eye is relatively insensitive they can be considered of no importance to visual colour. The band in the blue, related to curing practice, and extending from 460 mμ to 520 mμ, can be separated by two filters which cut off at these wave lengths. The remainder of the visible spectrum can then be divided into two regions, namely, those of haemoglobin and methaemoglobin absorption, by a third filter cutting off at 595 mμ.

In this way an instrument can be obtained to measure definite characteristics of colour quality and brightness independently, yet the method is as simple and rapid in operation as the original three filter method. This method may also be made suitable for colour measurement of paints, dyes, and textiles, etc. by using appropriate filters as determined by the above methods.

Acknowledgments

The author wishes to thank Dr. L. E. Howlett, Division of Physics and Electrical Engineering, National Research Laboratories, for the loan of the spectrometer, to express gratitude to Dr. W. H. Cook and Dr. J. W. Hopkins, Division of Applied Biology, National Research Laboratories, for their advice in the interpretation of the results, and to thank Mr. H. Tessier, Laboratory Assistant, for aid with the experimental measurements.

References

1. Brooks, J. Proc. Roy. Soc. (London) B, 123 : 368-382. 1937.
2. Brooks, J. Food Research, 3 : 75-78. 1938.
3. Cook, W. H. Can. J. Research, D, 19 : 85-95. 1941.
4. Cook, W. H. and White, W. H. Can. J. Research, D, 19 : 53-60. 1941.
5. Sidwell, A. E., Jr., Munch, R. H., Barron, E. S. G., and Hogness, T. R. J. Biol. Chem. 123 : 335-350. 1938.
6. Winkler, C. A. Can. J. Research, D, 17 : 1-7. 1939.
7. Winkler, C. A. Can. J. Research, D, 17 : 29-34. 1939.
8. Winkler, C. A., Cook, W. H., and Rooke, E. A. Can. J. Research, D, 18 : 435-441. 1940.
9. Winkler, C. A., Cook, W. H., Rooke, E. A., and Chadderton, A. E. Can. J. Research, D, 19 : 22-27. 1941.
10. Winkler, C. A. and Hopkins, J. W. Can. J. Research, D, 18 : 211-216. 1940.
11. Winkler, C. A. and Hopkins, J. W. Can. J. Research, D, 18 : 289-299. 1940.
12. Winkler, C. A., Hopkins, J. W., and Rooke, E. A. Can. J. Research, D, 18 : 225-232. 1940.
13. Winkler, C. A., Hopkins, J. W., and Thistle, M. W. Can. J. Research, D, 18 : 217-224. 1940.
14. Woodcock, A. H. and White, W. H. Can. J. Research, D, 21 : 85-89. 1943.

BEHAVIOUR AND NATURE OF THE FLUORESCING SUBSTANCES IN DRIED EGG POWDERS[1]

By J. A. Pearce[2]

Abstract

Fluorescence increased during storage in defatted, dried egg powders, and also in separately dried yolk and white. A portion of the fluorescent material was soluble in fat solvents and especially in alcohols. Ethanol may also destroy some of the fluorescing substance. The results indicate that fluorescent materials arise from changes in the protein fraction, that there is more than one fluorescing substance, and that some of these materials contribute to the increased fluorescence associated with decrease in quality. Indirect evidence indicates that proteose and peptone constituents are partly responsible for fluorescence, and that the nature of the deterioration is hydrolytic. A preliminary study was made of the effect of certain enzymes and micro-organisms on the formation of fluorescent materials. The work is being continued.

Introduction

The fluorescence of a potassium chloride extract of defatted dried egg powder was found to be closely correlated with the quality of the powder (3, 6). The urgent need of such a measurement delayed any detailed investigation of the fluorescent materials. The present investigation was concerned with the determination of the nature and behaviour of the fluorescing substances.

Materials and Procedure

The range of egg powders used in part of this investigation were kindly supplied by Canada Egg Products Ltd., Montreal.

The procedure used was essentially that previously described (3). However, in certain instances, fat and protein solvents other than chloroform, and 10% potassium chloride solution were used.

The results were recorded, as before, in photofluorometer units, but the variety of solutions used necessitated blank corrections to permit comparison of the results. Such corrections can be readily made since the scale of the Coleman photofluorometer is arranged to give a linear relation between scale reading and concentration of fluorescent material (cf. 3, Table I).

Source of Fluorescent Materials

An indication of the source of the fluorescent material was obtained by storing whole and chloroform defatted egg powder at 23.9° C. (75.0° F.). The results (Table I) show that fluorescence developed in egg powder regardless of the presence or absence of chloroform soluble components and indicated that changes in the protein fraction yield the fluorescing material.

[1] *Manuscript received February 22, 1943.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 89 of the Canadian Committee on Food Preservation and as N.R.C. No. 1112.

[2] *Biochemist.*

TABLE I

CHANGE IN FLUORESCENCE OF WHOLE AND CHLOROFORM DEFATTED EGG POWDER AT 23.9° C.

Material	Fluorescence reading[1] after storage for		
	0	Two months	Four months
Whole egg powder	27.0	40.3	69.0
Defatted egg powder	27.1	39.8	65.0

[1] *No blank corrections.*

If these changes are occurring in the protein fraction they may be common to proteins present in either the white or the yolk or both. To investigate this, yolks of fresh eggs were separated from the white and a small portion of the centre of the yolks carefully removed with a pipette. White, yolk, and the remaining yolk membrane with its adhering white were vacuum–ice dried and stored at 23.9° C. Table II shows the fluorescence readings after two

TABLE II

CHANGE IN FLUORESCENCE OF DRIED EGG YOLK, WHITE, AND MIXED YOLK AND WHITE, AT 23.9° C

Material	Fluorescence reading[1] after storage for		
	0	One month	Two months
Yolk	10.0	15.0	20.4
White	10.1	20.5	32.2
Mixed yolk and white	14.8	16.5	23.5

[1] *No blank corrections.*

months' storage. Initially yolk and white had approximately the same fluorescence. After storage the fluorescence of the dried white greatly exceeded that of the yolk, but the development of fluorescence was common to both dried white and dried yolk. Since there is very little fat in dried egg white, this is further indication that fractions other than fat are involved in deterioration.

Effect of Fat Solvents

The differential effect of fat solvents on fluorescence readings (3) may result from the presence of differentially soluble fluorescent materials, or may be due to solution or destruction of the fluorescing substance. The effect of fat solvents was therefore reinvestigated, with a view to clarifying the point. The pronounced reduction in fluorescence reading resulting from the use of ethanol (3) was observed for other alcohols and for glacial acetic acid (Table III).

The effect of ethanol as a solvent was studied. For this purpose egg powders with a range of quality were extracted with chloroform, and then with three 50 ml. portions of 95% ethanol. The ethanol extract was made up to 250 ml. and the fluorescence measured.

TABLE III

EFFECT OF FAT SOLVENTS ON FLUORESCENCE READING

Solvent	Fluorescence reading[1]
Glacial acetic acid (washed subsequently with chloroform)	1.0
Ethanol (abs.)	5.0
Isobutanol (sat. with water)	5.6
Methanol	6.0
Ethanol (95%)	6.8
Isobutanol	16.0
Chloroform[2]	36.8
Petrol ether	42.0
Acetone	43.0
Benzene	44.0
Ethyl ether	50.2
Toluene	54.5

[1] *Readings corrected for solution blank in all tables unless noted.*

[2] *Standard method (3).*

The logarithm of the photofluorometer readings of the alcohol extract plotted against computed taster score are compared with other curves in Fig. 1. It is apparent that a large portion of the fluorescent material was soluble in 95% ethanol. This material, after evaporation of the alcohol *in vacuo* at room temperature and dispersion in water, continued to fluoresce, but to a smaller extent than in alcohol. The dried material obtained from alcohol extraction was a pale yellow, amorphous solid. Although the fluorescence of the alcohol extract increases with decreasing quality, the difference in slopes of the curves for water and alcohol extraction indicates that alcohol soluble fluorescent material contributes less to the fluorescence of poor quality powders than it does to powders of good quality.

The fluorescence readings of the residual powder, after defatting with chloroform and extraction with alcohol, compare favourably with values previously observed when defatting was done with 3 : 1 petrol-ether–absolute-alcohol and extraction with 10% potassium chloride solution (3).

If solubility in the fat solvent were the only factor causing differences in fluorescence reading, it would be expected that the addition of fluorescence readings for the powder after alcohol extraction (Curve 4) and the fluorescence reading of the alcohol extract materials in water (Curve 3) would approximately equal the fluorescence readings in water (Curve 1). This is not

the case; therefore, it might be concluded that in addition to alcohol soluble and insoluble fluorescing substances, there are fluorescent materials destroyed by alcohol. Thus, the presence of more than one fluorescing substance is indicated.

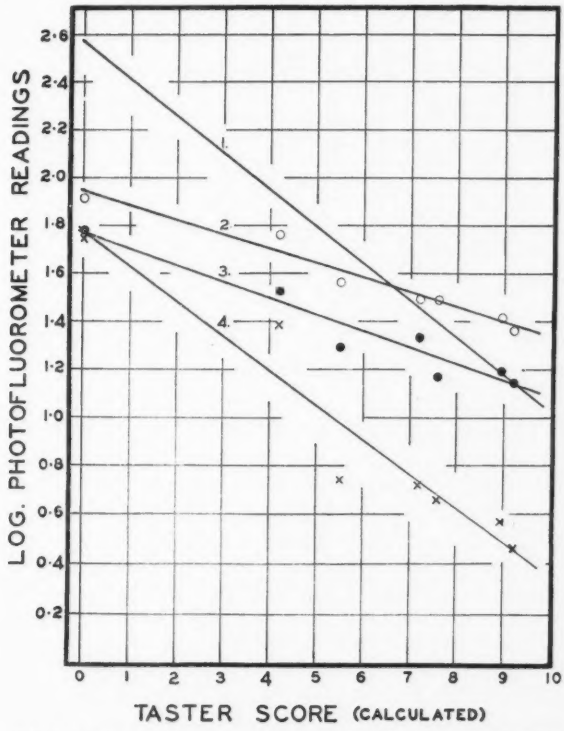


FIG. 1. *Effect of ethanol extraction on fluorescence reading. 1. Water extract of defatted powder. 2. Ethanol (95%) extract of defatted powder. 3. Water extract of alcohol soluble substance. 4. Potassium chloride (10%) extract of residue after ethanol extraction of defatted powder.*

Fluorescing Fractions

Lyophilic sols are known to fluoresce (2, p. 89). This phenomenon has been observed to occur in protein sols (5). Amino acids, the end products of protein decomposition, do not fluoresce (5). Foregoing work (3) indicates that the amount of fluorescence occurring in 10% potassium chloride extract prepared from fresh egg powder was small. Since fluorescence increases with decrease in powder quality, the highly fluorescent materials must, therefore, be intermediate products in protein decomposition.

Confirmation of this was sought by measuring the fluorescence of 1-gm. samples of commercial proteose–peptone mixtures (Difco), dispersed in 250 ml. 10% potassium chloride solution. These materials were found to have high

fluorescence values (Table IV). The values varied directly with free amino nitrogen content and pH and inversely with the diamino nitrogen and cystine content, as recorded by the manufacturer's analysis (1, p. 166). The full significance of these relations is not immediately evident, although the inverse relation with cystine content may point to breakdown of fluorescing substances to non-fluorescing amino acids as deterioration increases.

TABLE IV

FLUORESCENCE OF 0.4% PROTEOSE–PEPTONE MIXTURES IN 10% POTASSIUM CHLORIDE SOLUTION

Material	Fluorescence reading
Bacto–tryptone	216.7
Bacto–peptone	119.8
Proteose–peptone	83.4

Egg white is believed to contain about 80% ovalbumin, 10% ovomucoid, and 6.7% ovoglobulin, while egg yolk contains lecithin, lutein, cholesterol, vitellin, and livetin, the lecithin and vitellin probably existing in combination (4, p. 432). Of these, ovalbumin, ovomucoid, and livetin are soluble in water; ovalbumin, ovoglobulin, and lecithovitellin are soluble in dilute salt solutions. Proteoses and peptones are the protein decomposition products likely to be measured as they are soluble in water and dilute salt solutions; acid and alkali metaproteins and proteans are insoluble in these solutions.

Effect of Protein Solvents and Precipitants

The effect on the fluorescence reading of replacing 10% potassium chloride with 10% solutions of various salts and with protein precipitants (asbestos as a filter aid was omitted) is shown in Table V. Indications of the presence of any particular fluorescing protein are somewhat contradictory, again implying the presence of a number of fluorescing protein fractions. There are, also, indications of a lyotropic effect on the fluorescing substances; further evidence of their colloidal nature (7, p. 345).

Measurements were then made on a series of nine samples of dried egg powders with a range of quality to see if the fluorescing fractions were related to edibility. In addition the effect of filter aids on fluorescence readings was evaluated.

The mean fluorescence readings for each treatment together with an analysis of variance are given in Table VI. The very small fluorescence value in tannic acid solution was obtained with a sample of mixed milk and egg powder included in this series. Since all but this one reading were zero, these values were not included in the analysis of variance. The statistical analysis shows more fluorescing substances present in water extracts than in

TABLE V

EFFECT OF PROTEIN SOLVENTS AND PRECIPITANTS ON FLUORESCENCE READING

Solvent or precipitant	Fluorescence reading	Solvent or precipitant	Fluorescence reading
Sodium acetate	27.2	Sodium acetate	27.2
Sodium chloride	49.1	Lead acetate	8.6
Sodium nitrate	49.0		
Sodium sulphate	40.6	Sodium nitrate	49.0
Trisodium phosphate	23.4	Silver nitrate	0
		Cupric nitrate	0
Sodium chloride	49.1	Mercuric nitrate	0
Potassium chloride[1]	49.2		
Ammonium chloride	56.2	Picric acid (sat.)	0
Magnesium chloride	54.4	Tannic acid (0.5%)	0
Calcium chloride	40.0	Potassium ferrocyanide and	
Ferric chloride	0	acetic acid	0
		Trichloroacetic acid	8.6
Ammonium sulphate	52.0	Ammonium sulphate (sat.)	13.0
Sodium sulphate	40.6	Magnesium sulphate (sat.)	23.0
Magnesium sulphate	40.2		
		Water	41.8

[1] *Standard method (omitting asbestos as filter aid).*

TABLE VI

EFFECT OF TREATMENT ON FLUORESCENCE READINGS OF EGG POWDERS OF DIFFERENT QUALITY

Treatment	Mean fluorescence reading	Treatment	Mean fluorescence reading
Water	70.5	Magnesium sulphate (sat.)	46.9
Water and asbestos	64.7	Lead acetate (0.5 *M*)	40.4
Water and Fuller's earth	51.6	Sodium chloride (sat.)	38.8
Potassium chloride (10%)	51.2	Ammonium sulphate (half sat.)	5.4
Potassium chloride (10%) and		Ammonium sulphate (sat.)	4.5
asbestos	54.3	Tannic acid (0.5%)	0.03

Necessary difference, 5% level = 5.2.

Analysis of variance

Variance attributable to:	D.f.	Mean square
Samples	8	41987**
Treatments	9	23190**
Samples × treatments	72	348**
Duplicate error	82	50

** *Exceeds 1% level of statistical significance.*

10% potassium chloride and saturated magnesium sulphate extracts while the latter contained more than 0.5 *M* lead acetate or saturated sodium chloride. Ammonium sulphate solutions extract very little and tannic acid solution extracts no fluorescent material. Fig. 2 shows representative curves of those extractions that showed statistical differences.

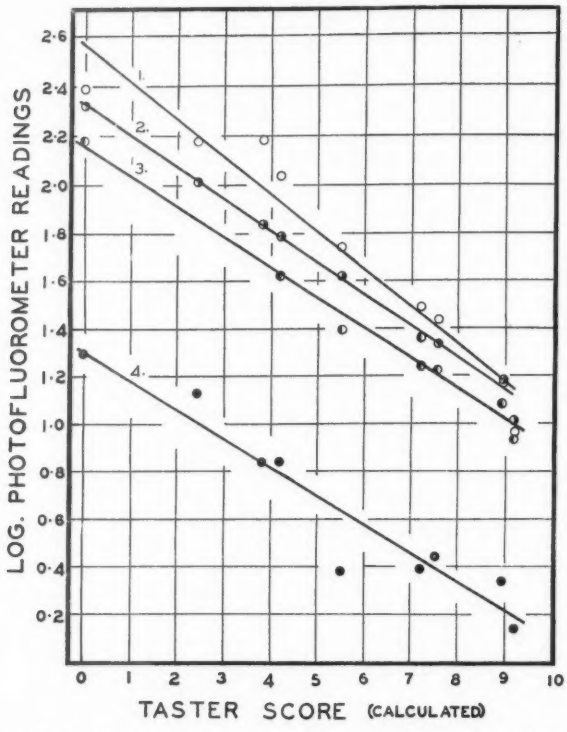


FIG. 2. *Effect of protein solvents and precipitants on fluorescence reading. 1. Water extract. 2. Potassium chloride (10%) extract. 3. Sodium chloride (saturated) extract. 4. Ammonium sulphate (half saturated) extract.*

The higher fluorescence values and greater range of readings obtained with water extraction are of no value in improving the method, because of extended filtering periods and poor agreement between duplicates. The use of sodium chloride, however, offers distinct practical advantages and is under investigation.

The results using filter aids showed that asbestos reduced fluorescence readings of water extracts, but made no significant change in readings when 10% potassium chloride solution was used. Fuller's earth did not remove the fluorescing substances completely from the water extracts, although it had been reported (5) to do so for other protein sols.

Effect of Heat on Formation of Fluorescing Substances

Water extracts of chloroform defatted powders were heated for 20 min. in a water-bath at 100° C. The presence of heat-coagulable fluorescent substances, or accelerated decomposition to non-fluorescing amino acids (5) should result in decreased fluorescence readings. On the other hand hydrolysis of non-fluorescing proteins to lower, fluorescent products should increase the readings. The latter case proved to be the more important, as shown by the increases noted in Table VII*a*. Partial fractionation of the heated extracts (Table VII*b*) supports the deduction that the nature of the change is hydrolytic. Further support is indicated by the effect of 1 *N* acids and bases (Table VIII) and by the work of others in these laboratories (8).

TABLE VII*a*

EFFECT OF HEATING WATER EXTRACTS OF DEFATTED, DRIED EGG POWDER

Treatment	Mean fluorescence reading
Water extract	88.4
Water extract, heated	116.1

Analysis of variance

Variance attributable to:	D.f.	Mean square
Samples	8	29343**
Treatments	1	6900**
Samples × treatments	8	544
Duplicate error	18	469

** *Exceeds 1% level of statistical significance.*

TABLE VII*b*

FRACTIONATION OF HEATED AND UNHEATED WATER EXTRACTS WITH AMMONIUM SULPHATE

Treatment	Mean fluorescence reading
(i) Unheated	
Ammonium sulphate (half saturated)	6.4
Ammonium sulphate (saturated)	5.3
(ii) Heated	
Ammonium sulphate (half saturated)	14.6
Ammonium sulphate (saturated)	12.2

Necessary difference, 5% level = 1.3.

Analysis of variance

Variance attributable to:	D.f.	Mean square
Samples	8	388**
Treatments	3	362**
Samples × treatments	24	12.3**
Duplicate error	36	3.46

** *Exceeds 1% level of statistical significance.*

TABLE VIII

EFFECT OF ACIDS AND BASES ON FLUORESCENCE READING

Material	Fluorescence reading in:		
	Water	1 *N* HCl	1 *N* NaOH[1]
Fresh egg powder	9.9	27.5	7.0
Old egg powder	35.0	27.5	11.5

[1] *Glass wool used for filtering.*

Effect of Enzymes and Bacteria on Formation of Fluorescing Substances

Hydrolysis as a feature of dried egg decomposition may be accelerated by the presence of enzymes. This was investigated by measuring the changes in fluorescence reading of 1% water sols of defatted egg powder containing 0.1 gm. of pepsin, papain, and trypsin per 100 ml. egg sol when incubated at 37° C. Increments in fluorescence readings greater than those occurring in the control were observed for papain and trypsin, as noted in Table IX. These measurements were difficult to make as enzymes in water have large fluorescence readings that decreased on incubation. It was impossible to determine whether or not the same decrease in fluorescence of enzymes occurred in the egg–enzyme mixtures.

TABLE IX

INCREMENTS IN FLUORESCENCE READINGS OF SOLUTIONS OF DEFATTED EGG POWDER IN WATER CONTAINING ENZYMES (pH = 6.9)

Treatment	Increment in fluorescence reading during incubation at 37.0° C.[1]			
	After 12 hr.		After 24 hr.	
Pepsin	−5.4	(+1.8)	4.6	(19.0)
Papain	14.0	(14.0)	18.2	(18.2)
Trypsin	6.4	(16.4)	26.4	(46.4)
Control	1.1		12.5	

[1] *Numbers in parentheses indicate values if decrement in fluorescence of enzyme solutions can be applied to enzyme–egg mixtures.*

No correlation between fluorescence reading and total bacterial count was apparent in previous work (6). However, a preliminary investigation, using defatted, dried egg in water as a medium for several strains of micro-organisms isolated from whole egg powder, showed that growth of certain of these organisms caused an increase in fluorescence reading*.

**Measurements made in the course of work (unpublished) by Mr. C. O. Fulton of these laboratories.*

Since there is a need to improve the keeping quality of dried whole egg powder, possible methods of retarding protein deterioration are under investigation.

References

1. DIFCO MANUAL of dehydrated culture media and reagents. 6th ed. rev. Difco Laboratories Inc., Detroit. 1939.
2. GORTNER, R. A. Outlines of biochemistry. 2nd ed. John Wiley and Sons, Inc., New York. 1938.
3. PEARCE, J. A. and THISTLE, M. W. Can. J. Research, D, 20 : 276-282. 1942.
4. PLIMMER, R. H. A. Practical organic and bio-chemistry. New ed. Longmans, Green and Co., Ltd., London. 1926.
5. REEDER, W. and NELSON, V. E. Proc. Soc. Exptl. Biol. Med. 45 : 792-794. 1940.
6. THISTLE, M. W., PEARCE, J. A., and GIBBONS, N. E. Can. J. Research, D, 21 : 1-7. 1943.
7. THOMAS, A. W. Colloid chemistry. McGraw-Hill Book Company, Inc., New York. 1934.
8. WHITE, W. H. and THISTLE, M. W. (In preparation).

CANADIAN JOURNAL OF RESEARCH

Notes on the Preparation of Copy

General:—Manuscripts should be typewritten, double spaced, and the *original* and *one copy* submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. All pages should be numbered.

Abstract:—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.

Illustrations

(i) *Line Drawings:*—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in *blue* only. Paper ruled in green, yellow, or red should not be used. The principal co-ordinate lines should be ruled in India ink and all lines should be of sufficient thickness to reproduce well. Lettering and numerals should be of such size that they will not be less than one millimetre in height when reproduced in a cut three inches wide. If means for neat lettering are not available, lettering should be indicated in pencil only. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. The *original drawings* and one set of small but clear *photographic copies* are to be submitted.

(ii) *Photographs:*—Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted *in duplicate;* if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:*—The author's name, title of paper, and figure number should be written on the back of each illustration. Captions should not be written on the illustrations, but typed on a separate page of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). Reference to each figure should be made in the text.

Tables:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. Reference to each table should be made in the text.

References should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper. The form of literature citation should be that used in this Journal and titles of papers should not be given. All citations should be checked with the original articles. Each citation should be referred to in the text by means of the key number.

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.

V
1
4
A
R
4
U